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14. ABSTRACT: In the final fifth year, we finalized research projected for all five Specific Aims of the grant. In the Aim 1, we have analyzed the role of geometry of anti-CAM conjugates in their targeting to endothelial cells. In the Aim 2, we have characterized the roles of mode of GOX targeting, oxygen supply and biological factors controlling acute lung injury in the new mouse model developed in this grant. In the Aim 3, we characterized protective effects of targeting antioxidant enzymes in animal model of oxidative stress caused primarily by H2O2 vs superoxide anion and found that targeting of catalase vs SOD provides effective protection in these cases, respectively. In the Aim 4, we completed characterization of thrombin-activated mutant fusion protein, scFv/uPA-T synthesized in the year 4. In the Aim 5, we have studied protective effects of anti-CAM/AOE and scFv/uPA-T in a mouse model of in situ lung ischemia/reperfusion, established in our group and found that targeting of both antioxidants into endothelium and pro-urokinase to endothelial surface protect the lung against oxidative and thrombotic stress in this clinically relevant model. Taken together, the data accumulated in the course of this grant and analyzed in the final report indicate that targeting of antioxidant and anti-thrombotic enzymes to endothelial CAMs affords significant advantages in treatment of pulmonary oxidative stress and thrombosis intertwined hallmarks of ALI/ARDS					
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Targeting of Drugs to ICAM-1 for Treatment of Acute Lung Injury

A. Introduction. The main goal of the project was to explore, test, optimize and prepare for translation into the clinical domain a new means for treatment of the Acute Lung Injury (ALI). In order to cope more effectively with oxidative and thrombotic stresses in the pulmonary vasculature, hallmarks of ALI, we designed targeted delivery of antioxidant enzymes (AOE) and fibrinolytic plasminogen activators (PA) to the endothelial cells lining blood vessels in the lungs. Targeting is provided by conjugation of AOE and PA with monoclonal antibodies directed to endothelial surface determinants, cell adhesion molecules ICAM and PECAM (collectively CAM) up-regulated and involved in ALI. Results of our animal studies imply that such targeted intervention may improve treatment of ALI.

During the five years of the study, we followed the proposed research plan and have completed five proposed Specific Aims: 1) Characterize ICAM-directed immunotargeting strategies and define the sub-cellular localization of the drugs; 2) Establish animal models of ALI; 3) Determine antioxidant protection by anti-CAM/AOE; 4) Determine fibrinolysis by anti-CAM/urokinase targeted to endothelial surface; 5) Evaluate the therapeutic potential of anti-thrombotic and anti-oxidant drugs immunotargeting to CAM in animal models of ALI.

By the present final report we indicate that research projected for these Specific Aims is completed. Implementation of this grant provided both new important basic science results including understanding of cellular and molecular mechanisms controlling sub-cellular targeting of CAM-directed drugs in endothelial cells, as well as design and successful testing and optimization of a new class of targeted therapeutic agents, which features are highly encouraging for use in treatment of ALI/ARDS and, most likely, many other vascular disorders that involve thrombosis, oxidative stress and inflammation. These important new results have been and continue to be published in numerous peer-reviewed journals. Of particular importance, a new series of recombinant protein constructs fusing anti-CAM scFv with mutant forms of urokinase plasminogen activator represents a good candidate pro-drug for industrial development and clinical studies. We actively pursue this avenue by seeking industrial partners and funds for scaling-up production, quality control and toxicological studies of this protein.

In the fifth year of the grant (a no-cost extension of the original four-year project) we concentrated our efforts both on new experiments aimed at completion and extension of the research plan, and on in-depth analysis, organization, summation and preparation for publishing of the entire body of the data accumulated in course of five-year study. Thus we produced a comprehensive analytical overview of the grant outcomes, necessary to put this study in the perspective. Based on this analysis, in the final year of the grant we performed new studies in all Specific Aims, in order to complete all main leads found in the course of the study. Accordingly, the summary report presented below briefly outlines main results obtained in the Aims throughout the study. Comprehensive description of the results, **highlighted by red color labels**, is presented as either references to specific figures and tables in the appendices or data presented in the previous reports, or included in the text as figures and tables.

B. Body of the Report.

B.1. Specific Aim 1. Characterize ICAM-directed immunotargeting strategies and define the sub-cellular localization of the drugs. In the framework of this Aim, we focused on: i) Synthesis and characterization of anti-CAM conjugates; ii) Understanding of mechanisms of their endothelial uptake; iii) Understanding of their intracellular metabolism in endothelium; iv) Quantitative characterization of their targeting to the pulmonary endothelium; and, v) Analysis of the role of size and shape of the anti-CAM conjugates in targeting to the pulmonary endothelium.

B.1.1. Synthesis, characterization and optimization of anti-CAM conjugates and fusion constructs. We systematically characterized these formulations in terms of their synthesis (streptavidin-biotin conjugation, SATA-SMCC chemical cross-linking, recombinant fusion techniques), purification (HPLC and affinity chromatography), resultant structure (SDS-PAGE, Western-blotting), size (using DLS and electron microscopy), antigen-binding activity (using RIA and ELISA), enzymatic activity (using synthetic and natural substrates of AOE and PA) and stability (using all above tests). We determined how these readouts are controlled by extent of chemical modifications of the conjugate ingredients (e.g., using HABA reagent for biotinylated AOE and anti-CAM), their molar ratio in the reaction, addition of protective and stabilizing agents (glycerol). Thus we established procedures for design, synthesis, purification and storage of anti-CAM/AOE and anti-CAM/PA conjugates and fusion proteins with optimal features (size ~300 nm and <10 nm respectively), with well preserved CAM-binding and enzymatic activities [1]. These formulations are available to our studies from stocks or producing cell lines. In the fifth year, we designed recombinant DNA for anti-PECAM/1-CysPrx fusion construct.

Comprehensive description of the results:

Figure 1 in appendix 1 shows the electron microscopy image revealing the size (diameter approx 200-300 nm) and polymorphous structure of the anti-CAM/catalase conjugate produced by streptavidin-biotin cross-linker.

Figure 2 in appendix 1 shows that size of the conjugates within the range of diameter of 100-400 nm can be accurately controlled by the molar ratio between biotinylated anti-CAM and catalase and streptavidin.

Figure 4 in appendix 1 shows that gel-filtration separates the conjugate from non-cross-linked conjugate components reveals high (~90%) yield of the conjugation procedure.

Figure 4B in appendix 1 shows that fluorescent microscopy reveals that anti-CAM/catalase, but not IgG/catalase conjugate specifically binds to endothelial cells.

Figure 5 in appendix 1 shows that anti-CAM/catalase conjugate enhances H₂O₂-degrading capacity of the endothelial cells (panel A) and effectively protects endothelial cells from cytotoxic effect of hydrogen peroxide (revealed by release of ⁵¹Cr, panel B, and by preserved cellular morphology, panel C).

Figure 6 in appendix 1 shows that anti-CAM/catalase conjugate retains its size and enzymatic activity during 72 hours storage in glycerol at -20°C. The inset shows conjugate stability under these conditions during 12 months.

Figure 7 in appendix 6 shows that size of anti-CAM/SA conjugates, determined by dynamic light scattering (DLS) and fluorescent microscope can be controlled in the range 100-300 nm or 1,000-10,000 nm.

Figure 2 in appendix 9 shows that anti-CAM conjugates bind specifically to both static and flow-adapted endothelial cells under flow.

Figure 3 in appendix 9 shows that anti-CAM conjugates bind to endothelial cells in the intact perfused mouse vasculature in situ.

Figure 5 in appendix 9 shows that affinity of binding to endothelial cells of multivalent anti-CAM conjugates is 100 times higher than that of unconjugated anti-CAM.

B.1.2. Regulation of internalization of anti-CAM conjugates by endothelial cells. We have found that it is possible to deliver therapeutics to either surface or interior compartments of the endothelium by controlling key features (first of all, size) of anti-CAM conjugates. The novelty of this concept is exquisite, since no prior knowledge existed on whether endothelial cells (EC) internalize materials anchored to CAMs and how the fate of these materials is regulated. Accordingly, understanding of molecular and cellular mechanisms regulating uptake of the cell-anchored anti-CAM conjugates is of paramount importance for the fields of drug delivery/experimental therapeutics and vascular biology. Below we briefly outline the major mechanistic findings of the grant.

EC do not internalize monomeric anti-ICAM regardless of cytokine activation of anti-ICAM binding. Using labeled anti-ICAM, FACS and fluorescent microscopy, we found that EC internalize no more than 10% of cell-bound anti-ICAM. Pro-inflammatory cytokines augment anti-ICAM binding to, but not internalization by EC.

Endothelial cells internalize small, but not large anti-CAM/conjugates. Using anti-CAM conjugates of controlled size, we showed that EC internalized conjugates within a diameter of 100-200 nm, but did not internalize conjugates larger than 0.5 μ m [2].

Molecular mechanism of endothelial uptake of anti-CAM conjugates. We studied the molecular mechanism regulating anti-CAM conjugates uptake in endothelium and found that it represents a previously unknown endocytic pathway, which we named CAM-mediated endocytosis, characteristic of both ICAM-1 and PECAM-1. Using mutant cell lines, pharmacological inhibitors, anti-sense oligonucleotides and diverse analytic techniques including tracing of labeled anti-CAM conjugates of given size in fluorescent and electron microscope, we defined the unique features of this pathway. They include: i) key role of actin and its adaptor, dynamin, in the formation of endocytic vesicle, initiated by cross-linking of CAM in the plasma membrane and regulated by amiloride-sensitive membrane ion exchanger, NHE-1; ii) CAM-endocytosis neither co-localize with caveoli, clathrin, phagocytic and pinocytosis pathways, nor gets affected by inhibitors of these pathways; iii) CAM-endocytosis is coupled with elevation of cytosolic calcium and is regulated by protein kinase C, ROCK-Rho kinases, but not PI3-kinases [3].

Comprehensive description of the results:

Figure 1 in appendix 2 shows that cultured endothelial cells do not internalize monomolecular anti-CAM and internalize anti-CAM/conjugates with diameter <100-300 nm, but not >500 nm, and that 50% internalization time for anti-PECAM and anti-ICAM conjugates is 15 and 30 min, respectively.

Figure 2 in appendix 2 shows that genetic mutation of dynamin inhibits endothelial internalization of anti-CAM conjugates

Figure 3 in appendix 2 shows that anti-CAM conjugates do not enter via cellular structures identified by caveolin and clathrin.

Figure 4 in appendix 2 shows that anti-CAM conjugates enter endothelial cells via the endocytic pathway that is resistant to inhibitors of clathrin- and caveolin-mediated uptake, yet sensitive to NHE-1 inhibitor, amiloride.

Figure 6 in appendix 2 shows that endothelial internalization of anti-CAM conjugates is inhibited by PKc inhibition and stimulated by PKc activation.

Figure 7 in appendix 2 shows that internalization of anti-CAM conjugates is associated with rearrangement of actin cytoskeleton in endothelial cells.

Figure 8 in appendix 2 shows that endothelial internalization of anti-CAM/conjugates is inhibited by antagonists of ROCK and Src kinases, but not PI-3 kinase and requires intact activity of actin-regulating cytoskeletal proteins.

B.1.3. Natural and pharmacological control of sub-cellular destination of CAM-targeted conjugates and their safety. Since CAM-medicated endocytosis is a truly unique and previously unrecognized pathway, the intracellular destination of conjugates their metabolism, fate and duration of activity was an enigma. We used multi-label fluorescent microscopy, electron microscopy and analysis of labeled conjugates cells to address this issue, critically important for effects of anti-CAM/AOE conjugates. In this section we briefly describe the cardinal findings.

Slow lysosomal traffic of the conjugates. Using labeled antibodies to endosomal and lysosomal markers EEA-1 and LAMP-1 we showed that after uptake, conjugates reside in early endosomes for 1-2 h and traffic slowly to lysosomal compartments by 3 hours, which coincides with degradation of protein cargo in lysosomes 3 h after uptake [4].

Role of NHE-1/NHE-6 molecular switch in lysosomal traffic of conjugates. By using antisense oligonucleotides, highly selective pharmacological inhibitors and multi-label fluorescent microscopy we found that traverse of anti-CAM conjugates from endosomal to lysosomal compartment is controlled by ion exchanger NHE-6 localized in the membranes of these vesicles [5].

Recycling to the plasma membrane. Using radioisotope tracing and fluorescent microscopy we found that CAM molecules co-internalized with anti-CAM conjugates dissociate from the latter in an NHE-6 controlled pre-lysosomal sorting compartment and recycle to the endothelial plasma membrane, thus permitting sustained intracellular drug delivery via CAM [6].

Pharmacological control of intracellular traffic. Chloroquine inhibits acidification of lysosomes and decelerates degradation of the conjugates without altering their

trafficking. Nocodazole, an agent which disrupts microtubules, blocked traffic of conjugates in the endosomal compartment and thus markedly prolonged stability of conjugates. Monensin, a drug that alters NHE-6 switch in the endosomes, diverts conjugates from lysosomal traffic and stimulates recycling of the internalized conjugates to the endothelial lumen, which markedly prolongs duration of therapeutic activity of the delivered AOE catalase [3-8].

Anti-CAM conjugates do not induce overt acute toxicity in endothelial cells. We tested whether intracellular accumulation of anti-CAM/conjugates damages EC. We found that: i) EC retain internalized non-degradable anti-CAM conjugates for many days without detectable cellular toxicity; ii) Loading of EC with saturating doses of anti-CAM conjugates does not block constitutive endothelial endocytosis and traffic; iii) Conjugate-loaded EC retain normal morphology and ability to divide. These data imply high safety of anti-CAM conjugates [6].

Comprehensive description of the results:

Figure 2 in appendix 3 shows double-label fluorescent microscopy of endothelial cells after internalization of anti-CAM conjugates using EEA-1 and LAMP-1 markers of endosomes and lysosomes and reveals that 60% of internalized conjugates are in endosomes at 1 h, whereas 80% of internalized conjugates are in lysosomes at 3 h.

Figure 3 in appendix 3 shows kinetics of degradation of anti-CAM protein conjugates and reveals that ~60% and 20% of internalized conjugate is stable 2 and 3 hours after the uptake.

Figure 4 in appendix 3 shows that chloroquine does not alter the intracellular traffic of anti-CAM conjugates, but precludes their lysosomal degradation (~75% of stable conjugate at 3 hours).

Figure 5 in appendix 3 shows that nocodazole blocks intracellular traffic of anti-CAM conjugates in endothelial cells prior to EEA-1 positive endosomal compartment.

Figure 8 in appendix 3 shows that protective effect of anti-CAM/catalase conjugate is observed at 1 and 2 hours after internalization by endothelial cells, but protection is gone by 3 h, whereas chloroquine and nocodazole prolong protective effect to 3 and 5 hours post-internalization, respectively.

Figure 1 in appendix 4 shows that inhibition by EIPA, a specific pharmacological antagonist of NHE-1, both prevents anti-CAM conjugates internalization in endothelial cells and actin cytoskeleton remodeling associated with this process.

Figure 2 in appendix 4 shows that inhibition of NHE-1 synthesis using specific siRNA also blocks these apparently linked processes.

Figure 3 in appendix 4 shows that NHE-1, but not NHE-6 clusters in the sites of binding and internalization of anti-CAM conjugates in the endothelial plasma membrane.

Figure 4 in appendix 4 shows that at 30 min and 60 min after binding to endothelial cells, anti-CAM conjugates are associated with NHE-1 and NHE-6 compartments, respectively.

Figure 5 in appendix 4 shows that monensin inhibits delivery of anti-CAM conjugates to the lysosomes, whereas NHE-1 inhibitor EOPA and PKc inhibitor H7 have no such effect.

Figure 1 in appendix 5 shows that ICAM-1 transiently disappears from the endothelial plasma membrane in course of CAM-mediated endocytosis of anti-ICAM nanoparticles.

Figure 2 in appendix 5 shows that although anti-ICAM conjugates traffic to the lysosomes after internalization by endothelial cells, co-internalized ICAM-1 recycles to the cell surface.

Figure 3 in appendix 5 shows that ICAM-1 recycling allows sustained binding and internalization of several sequential doses of anti-ICAM conjugates by endothelial cells.

Figure 4 in appendix 5 shows that the subsequent doses of anti-ICAM/nanoparticles traffic to the endothelial lysosomes slower than previous doses, due to saturation of the endosomal trafficking pathway.

Figure 5 in appendix 5 shows that saturation of intracellular trafficking pathway by sequential doses of non-degradable anti-ICAM/nanoparticles does not cause adverse effects on traffic of fluid phase components in the endosomal-lysosomal compartment in the endothelial cells.

Figure 6 in appendix 5 shows that saturation of intracellular trafficking pathway by sequential doses of non-degradable anti-ICAM/nanoparticles does not adversely affect viability, proliferation and morphology of endothelial cells.

Figure 6 in appendix 4 shows that monensin that disrupts microtubules enhances recycling of anti-CAM conjugates to endothelial plasmalemma after internalization.

Figure 7 in appendix 4 shows that monensin markedly delays lysosomal degradation of anti-CAM conjugates internalized by endothelial cells and thus prolongs protective effect of anti-CAM/catalase by at least 6 hours.

B.1.4. Quantitative analysis of anti-CAM targeting to the pulmonary endothelium. We studied systematically targeting of anti-CAM to the pulmonary endothelium, using tracing of the antibodies, conjugates and fusion proteins perfused in isolated rat lungs and injected in naïve animals. For tracing of these agents, we utilized radioisotopes, fluorescent labels for intravital and tissue section fluorescent microscopy and electron microscopy. Below we briefly outline the major findings.

Quantitative analysis of anti-ICAM targeting in the perfused rat lungs. Using this experimental model that excludes systemic affects and clearance, we verified specific targeting of ^{125}I -anti-ICAM, but not IgG in the pulmonary vasculature reaching saturation at the level of $\sim 5 \times 10^{13}$ anti-ICAM binding sites per gram ($\sim 1.5\text{-}2.5 \times 10^5$ binding sites per endothelial cell by Scatchard analysis). By comparing uptake at 6°C vs 37°C and binding of secondary perfused ligands, we validated that EC do not internalize anti-ICAM in this model.

Pulmonary targeting of anti-CAM in intact animals. We found that anti-CAM and anti-CAM conjugates and fusion proteins, but not IgG counterparts, accumulate in the lungs after intravenous injection in rats and in mice. Pro-inflammatory factors (hyperoxia, endotoxin) markedly facilitated pulmonary uptake of anti-

ICAM, had little effect on pulmonary uptake of anti-PECAM and suppressed uptake of anti-ACE and anti-TM [9-11].

Comprehensive description of the results:

Figure 2 in appendix 6 shows that anti-ICAM accumulates specifically in the isolated perfused rat lungs with the quantitative binding parameters listed above.

Figure 4 in appendix 6 shows that specific pulmonary targeting of radiolabeled anti-ICAM injected IV in rats achieves 16% ID/g, localization ratio 45 and immunospecificity of 250.

Figure 5 in appendix 6 shows specific pulmonary uptake of radiolabeled anti-ICAM in rat lungs and that LPS further stimulates anti-ICAM targeting.

Figure 6 in appendix 6 shows that by fluorescent and electron microscopy, anti-ICAM binds in the lungs specifically to endothelial cells.

Figure 8 in appendix 6 shows that anti-ICAM conjugated tPA accumulates in rat lungs after IV injection.

Figure 5 in appendix 7 shows that radiolabeled anti-ICAM/tPA accumulates in the lungs after injection in rats either via venous or arterial route, similarly to unmodified anti-ICAM.

Figure 2 in appendix 8 shows that pulmonary targeting of antibodies directed to endothelial surface determinants and conjugates based on these antibodies is controlled by affinity of the antibodies.

Figure 4 in appendix 9 shows that anti-CAM, but not control IgG conjugates and nanoparticles accumulate in the lungs after IV injection in intact mice and show visualization of their specific endothelial targeting in vivo using fluorescent microscopy.

Figure 6 in appendix 9 shows that pulmonary targeting of multivalent anti-CAM conjugates injected IV in intact mice greatly exceeds that of non-conjugated anti-CAM.

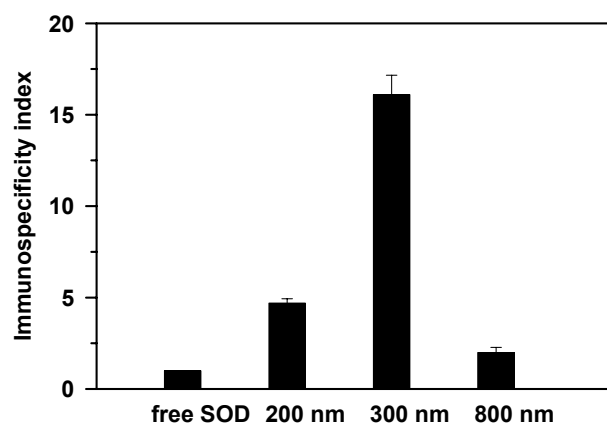
B.1.5. Control of the pulmonary targeting by size and shape of anti-CAM conjugates. Results of our in vitro studies in cell cultures clearly indicated that geometrical features (e.g., size) of anti-CAM conjugates control their sub-cellular targeting in endothelial cells. In order to complete this novel aspect of our study, i.e., role of the conjugate geometry in the targeting, in the fifth year of the grant we have studied how size and shape of the conjugates control their targeting to the pulmonary endothelium.

Optimal size for pulmonary targeting of polymorphous protein conjugates. We have compared pulmonary accumulation of radiolabeled anti-CAM/AOE conjugates with mean diameter 100, 300 and 1000 nm injected IV in mice. IgG/AOE conjugates of similar size were used in control to account for the non-specific uptake. We found that specific uptake was maximal for 300 nm diameter conjugates, exceeding that of 100 nm conjugates by 4-5 times. Absolute level of pulmonary uptake of large, micron size conjugates was even greater than that of 300 nm counterparts, but the specificity of the targeting was greatly reduced due to the fact that 1,000 nm diameter IgG/AOE conjugates showed high non-specific

uptake in the pulmonary vasculature (capillaries). Thus, coincidentally, optimal size for both intracellular delivery and pulmonary targeting of protein anti-CAM conjugates is ~300 nm.

Comprehensive description of the results:

Figure 1 shows that optimal size of anti-CAM nanoconjugates providing the most effective and specific targeting of SOD to the pulmonary vasculature (vs IgG-counterparts) is approximately 300 nm in diameter.



Diameter of anti-PECAMSOD conjugate

Shape controls pulmonary targeting of anti-CAM conjugates. Protein conjugates used in the previous study were prepared by strept-avidin cross-linking, which provides polyvalent polymorphous complexes. At the present time, there is no means to control the shape of these complexes. To get an insight into whether and how the shape controls targeting, we used model polystyrene beads of either spherical or elliptical shape, coated by anti-ICAM or IgG. Tracing of radiolabeled anti-ICAM/beads and IgG/beads after injection in mice revealed that discoid shape (similar to natural shape of red blood cells) favors targeting to pulmonary endothelium, likely due to alignment with flow patterns in the vasculature. These new studies, initiated in the last year of the grant, provide a basis for a new direction in drug delivery field, namely, investigation of the role of a drug delivery system geometry in targeting.

Comprehensive description of the results:

Table 1 shows non-specific uptake of nanocarriers of diverse shape in the pulmonary vasculature after IV injection in mice. IgG-coated latex discoid nanoparticles (**NP**) and filamentous worm micelles *wm* showed respectively a 2-fold and 6-fold higher blood level than spherical IgG **NP** 1h after IV injection in mice (Table 1), despite the much larger maximal dimension of the former carriers (discs and *wm* are 3 μ m and ~18 μ m in length, spheres \emptyset is 150 nm). IgG discs and IgG *wm* also showed less non-specific vascular uptake than spherical IgG **NP**, revealed by the retention in the pulmonary capillaries.

	IgG spheres	IgG discs	IgG <i>wm</i>
Blood, %ID	6.2 \pm 0.4	13.8 \pm 0.8	38.1 \pm 7.7
Blood, %ID/g	3.6 \pm 0.3	7.7 \pm 0.3	25.8 \pm 5.2
Lung, %ID/g	9.3 \pm 1.4	5.2 \pm 0.5	4.7 \pm 1.2
Lung/blood LR	2.7 \pm 0.4	0.7 \pm 0.1	0.2 \pm 0.03
Bloodless lung	ND	ND	1.1 \pm 0.7
Lung/blood LR	ND	ND	0.09 \pm 0.04

Table 1: Reduced blood clearance and pulmonary retention of elongated ¹²⁵I-IgG carriers (non-specific IgG). Mice were injected via tail vein with IgG-conjugated formulations and 1 hour later isotope level in blood and lung was measured to calculate % of injected dose per gram of tissue (%ID/g). Blood represents ~7% of body weight, i.e., ~1.5-1.8 g in adult 20-25 g mice. LR, localization ratio: %ID/g in an organ divided by %ID/g in blood. The data is M \pm SEM, n=3-5.

Since the major fraction of IgG *w/m* remains in the blood, the lung level of isotope largely reflects the residual blood pool in the pulmonary vessels (~30% of lung mass). Infusion of 10 ml PBS/heparin at sacrifice, providing bloodless white lungs, showed that indeed ~80% of IgG *w/m* in the lung harvested without this procedure belongs to residual blood (1.1 ± 0.7 vs 4.7 ± 1.2 %ID/g in perfused vs non-perfused lungs). No such correction was warranted for IgG spheres and IgG discs showing much lower blood levels. The difference requires that the blood level of IgG *w/m* in the lungs (i.e., $4.7 - 1.1 = 3.6$ %ID/g 1 h after injection) be subtracted from data on the pulmonary uptake of targeted *w/m* (Table 2, below).

Table 2 shows effect of the nanocarriers shape on specific targeting to the pulmonary vascular endothelium after IV injection in mice. To assess targeting, we injected the carriers bearing mAb to endothelial determinants ICAM-1, PV-1 and thrombomodulin (TM) traced by ^{125}I -IgG. The Ab surface density was $\sim 7,000$ Ab/ μm^2 for spheres and discs and $\sim 8,000$ Ab/ μm^2 for *w/m*. The ratio between lung level of Ab formulations and IgG counterparts (Immunospecificity Index, ISI) reflects specific endothelial targeting. These new data provide important insight into Ab affinity and carrier geometry effects.

Lung, % ID/g	mAb	mAb/spheres	mAb/discs	mAb/ <i>w/m</i>
IgG	11.3 ± 0.8 (ISI=1)	9.3 ± 1.4 (ISI=1)	5.2 ± 0.5 (ISI=1)	1.1 ± 0.7 (ISI=1)
Anti-ICAM-1	50.4 ± 2.3 (ISI=4.5)	114.7 ± 11.1 (ISI=12)	186.2 ± 15.4 (ISI=35.9)	11.7 ± 1.8 (ISI=11)
Anti-PV1	133.4 ± 12.4 (ISI=12)	25.8 ± 1.2 (ISI=2.8)	ND	ND
Anti-TM ₄₁₁	115 ± 3.6 (ISI=10)	349 ± 27 (ISI=38)	ND	18.8 ± 2.1 (ISI=17)
Anti-TM ₂₇₃	75 ± 2.0 (ISI=6.6)	39.3 ± 2.0 (ISI=4.2)	ND	4.2 ± 1.8 (ISI=3.8)

Table 2. Carrier geometry, and mAb affinity and their accessibility to EC determinants control targeting of nanocarriers to the pulmonary vasculature. Experimental conditions as in the previous Table. ISI: %ID/g of mAb to %ID/g of IgG, Immunospecificity Index. Residual blood pool level of *w/m* in the lungs (3.6%ID/g, Table 1) was subtracted from the data of anti-ICAM and anti-TM *w/m*.

B.2. Specific Aim 2. Establish animal models of ALI. A plethora of animal models have been developed for investigation of ALI. The models employing airway insults induce local injury, but do not adequately simulate events in the vascular compartment in ALI. The models employing intravascular insults better resemble variants of human ALI and are characterized by PMN recruitment and increased vascular permeability, but in these models pulmonary mechanisms manifestations and are overshadowed by systemic abnormalities. In order to test effects of anti-CAM conjugates and advance the field of experimental ALI/ARDS research, we studied antibody-directed vascular targeting of the H_2O_2 -generating enzyme glucose oxidase (GOX) to thrombomodulin (TM) normally expressed on the luminal surface of pulmonary EC. Thus we have established a new series of ALI models in mice, based on IV injection of anti-TM/GOX and documented that key features of these models closely resemble pathological features of human ALI and can be controlled by tuning such parameters as anti-TM affinity, oxygen level, level of TM expression in the lungs and animal gender [11,12].

B.2.1. Characterization of anti-TM/GOX model. Our results indicate that lung injury induced by anti-TM/GOX bears several hallmarks of human ALI syndrome: i) an acute oxidant stress in the pulmonary vasculature; ii) WBC influx in the lungs; iii) pulmonary thrombosis. In order to imitate clinical settings of ALI/ARDS

even closer, we have established a double-insult model combining hyperoxia and anti-TM/GOX insults and carefully characterized lung pathology in these models.

Pulmonary oxidative stress and thrombosis caused by anti-TM/GOX in mice. Anti-TM/GOX caused acute (within 2-4 hours), dose-dependent oxidative stress in the lung detected by markers of lipid and protein oxidation, dramatic elevation of protein level in the broncho-alveolar lavage fluid (BAL) and 85-95% mortality within 6 h post injection of high dose (2-2.5 ug/g anti-TM/GOX).

Inflammation and thrombosis in anti-TM/GOX model. Oxidative lung injury by anti-TMGOX is associated with influx of WBC to the lung, detected by MPO level in lung homogenates and ten-fold elevation of WBC number in the BAL by 24 hours after anti-TM/GOX injection. Deposition of fibrin and platelets aggregates were evident in the lungs of mice 4 hours post anti-TM/GOX injection.

Quantitative analysis of pulmonary oxidative stress in mice. We have established quantitative parameters of oxidative injury inflicted by GOX, hyperoxia or their combination including 8-epi isoprostane (determined by HPLC/MS) and MDA in homogenate showing lipid peroxidation, protein carbonyls showing protein oxidation and myeloperoxidase (MPO) showing PMN sequestration, protein and WBC in the BAL, showing edema and WBC (WBC) alveolar transmigration.

B.2.2. Dynamics of CAM exposure and WBC accumulation in the lungs. Of particular interest was to learn how anti-TM/GOX and hyperoxia alter expression of target CAM in the lungs and potential effect(s) of blocking WBC interactions with CAM in this model, in order to: i) further characterize this pathology model and help to understand its molecular and cellular mechanisms; ii) guide fine-tuning of design of anti-CAM conjugates, for example, built-in capacity to block CAM; and, iii) provide a basis for interpretation of protective effects of anti-CAM/AOE, such as contribution of blocking of secondary inflammation [11].

Elevated surface expression of CAMs in the lungs. Using tracing of ¹²⁵I-labeled mAbs against P-selectin and ICAM-1 injected in mice along with ¹³¹I-IgG that traces non-specific uptake in the organs, we found that anti-TM/GOX caused a 5-fold and 2-fold elevation of pulmonary vascular exposure of P-selectin and ICAM-1, respectively. Using similar technique, we also detected significant elevation of P-selectin and ICAM vascular exposure in the lungs of mice exposed to hyperoxia, indicating that: a) EC is a prime target of oxidant stress in these models; b) anti-CAM/AOE targeting likely to be elevated in these models; and, iii) CAM exposure may lead to secondary propagation of inflammation due to WBC adhesion.

Blocking of CAM does not protect against pulmonary injury. We tested role of CAMs in pulmonary WBC influx and tissue injury in anti-TM/GOX and hyperoxia models using: i) blocking CAM antibodies and combinations thereof, ii) neutrophil depletion; and, iii) CAM knockout mice. Either ICAM or PECAM antibodies but not P-selectin antibody attenuated pulmonary influx of WBC in hyperoxia, but failed alleviate lung injury (wet/dry ratio and protein levels in BAL). Similarly, PECAM-1, ICAM-1 and P-selectin KO mice failed to attenuate hyperoxic lung

injury. Finally, PMN depletion also failed to prevent lung injury in this model. Studies in anti-TM/GOX model produced similar results, namely, no protective effects of either blocking CAM antibodies or PMN depletion. PECAM KO mice showed significantly less % BAL WBC in BAL, but no difference in lung injury in anti-TM/GOX model. This outcome indicates that PMN play rather limited roles in propagating of acute phase of our models.

B.2.3. Modulation of the oxidative lung injury by anti-TM/GOX: selection of anti-TM carriers, model of combined anti-TM/GOX/hyperoxia and role of endogenous biological factors. In the last year of the grant, we studied how features of anti-TM/GOX conjugates and experimental conditions control the key pathological parameters of this model. We tested effects of: i) anti-TM affinity and composition of anti-TM/GOX; ii) level of the inhaled oxygen; iii) individual biological parameters of experimental animals [12].

Pulmonary targeting of anti-TM/GOX is controlled by affinity to TM. We have compared pulmonary uptake of several anti-TM/GOX conjugates, based on two TM monoclonal antibodies directed to distinct TM epitopes, conjugated to GOX either via streptavidin (conjugates size ~300 nm) or via co-immobilization on 100 nm polystyrene nanospheres. First, we quantified the pulmonary targeting 60 min after IV injection of ^{125}I -labeled anti-TM or each conjugate carrying tracer amounts (~1 $\mu\text{g}/\text{mouse}$) of ^{125}I -GOX. The control IgG, IgG/GOX and IgG/bead/GOX formulations that were used to account for non-specific uptake in the pulmonary vasculature showed no appreciable pulmonary uptake, i.e., lung-to-blood ratio <1. In contrast, both anti-TM antibodies showed strong pulmonary targeting with lung-to-blood ratios approaching ~75 for the best formulations. Despite effective targeting of either of the antibodies alone, targeting of the conjugates with the TM₃₄ antibody were far inferior to the targeting of conjugates made with the TM₂₀₁ antibody. Thus, anti-TM₂₀₁/ ^{125}I -GOX and anti-TM₂₀₁/bead/ ^{125}I -GOX showed pulmonary uptake that were an order of magnitude higher than the anti-TM₃₄/ ^{125}I -GOX and anti-TM₃₄/bead/GOX and achieved 349 ± 27 %ID/g for the best formulation, namely, anti-TM₂₀₁/bead/ ^{125}I -GOX. Thus, antibody affinity and structure of conjugates control their targeting [12].

Injurious effect of anti-TM/GOX is controlled by affinity to TM and GOX activity. Consistent with more effective targeting, anti-TM₂₀₁/GOX caused more severe lung injury vs anti-TM₃₄/GOX at doses ranging from 0.75 $\mu\text{g}/\text{g}$ to 1.5 $\mu\text{g}/\text{g}$, manifested by edema, leukocyte infiltrate and alveolar damage. In fact, anti-TM₂₀₁/GOX caused ~5-fold higher elevation of BAL protein level vs anti-TM₃₄/GOX, which correlates well with the targeting difference of conjugates. On the other hand, effect of anti-TM/GOX depends on its enzymatic activity. Both streptavidin anti-TM/GOX conjugates retained ~60% of GOX activity. In contrast, absorption to polystyrene beads caused a 96% GOX inactivation, which is why anti-TM₂₀₁/bead/GOX, which displayed the superior targeting features, caused only marginal lung injury even at the dose of 2 $\mu\text{g}/\text{g}$. Therefore, streptavidin anti-TM/GOX conjugates represent preferential tool for modeling pulmonary oxidative stress and ALI [12].

Double-hit injury model: hyperoxia augments injurious effect of anti-TM/GOX. To test the role of the oxygen supply in the effect of targeted GOX, we exposed mice to various levels of O₂, the substrate necessary for the enzymatic production of H₂O₂ by GOX. Five minutes after anti-TM/GOX injection, mice were placed in a chamber with regulated inflow of O₂. Neither low doses of anti-TM/GOX in room air (1 ug/g), nor 80-100% O₂ caused a significant lung injury in mice within 4 h. In fact, no detectable injury was found in mice exposed to hyperoxia alone for 24 h. Yet, combination of anti-TM/GOX and 100% O₂ caused acute severe oxidative lung injury detectable by 5-fold elevation of protein in BAL and doubling of MDA level in the lung homogenates. Aggravation of anti-TM/GOX injury by hyperoxia was manifested by elevated level of: i) BAL protein; ii) tissue injury revealed by histopathology evaluation and immunostaining for nitrotyrosine; and, iii) accumulation of the products of lipid peroxidation in the lung tissue. In contrast, hypoxia (10% O₂) significantly reduced the BAL protein level in anti-TM/GOX-treated animals to levels similar to basal amounts seen in naïve control mice, indicating that modulating effect of oxygen is due to regulation of the substrate supply for GOX [12].

Thrombomodulin level in lungs controls the targeting and effect of anti-TM/GOX. We noticed in several series that some mice were more resistant to anti-TM/GOX injury vs the majority of animals within the group. We hypothesized that these fluctuations might reflect, among other factors, individual variability in targeting of the conjugates and/or sensitivity to oxidative stress. It is known that TM level in the endothelium is reduced in response to many factors. We posited that some animals utilized in our anti-TM/GOX studies may have low TM levels in the lungs, due to undetected and uncontrolled variations in their health. We therefore used Western blotting to correlate levels of TM and targeted GOX in the tissue homogenates of lungs obtained from mice that displayed a wide range of variability of injury induced by anti-TM₃₄/GOX in several series and found that: i) TM levels in the lungs of "naïve" mice do vary substantially; ii) mice with low endogenous level of TM in the lungs showed proportionally lower targeting of anti-TM/GOX; iii) this, in turn resulted in a lower extent of lung injury induced by injected anti-TM/GOX [12].

Gender controls sensitivity to oxidative lung injury induced by anti-TM/GOX. Analyzing data of ~100 anti-TM/GOX experiments performed during five years we noticed anecdotal deviations in extent of lung injury in animals that differ from our main stock by age, breeder and strain. In theory, these factors can modulate susceptibility to GOX enzymatic product, H₂O₂. We tested systematically role of gender and found that male mice are more sensitive anti-TM/GOX injury vs female counterparts. Most likely, this outcome reflects higher sensitivity of male mice to oxidative injury, since male mice were more sensitive to oxidative injury induced by 72 hours exposure to hyperoxia [12].

Comprehensive description of the results:

Figure 1 in appendix 10 shows histological analysis of the kinetics of development of pathological alterations induced in the mouse lungs by exposure to hyperoxia (tissue edema, accumulation of leukocytes, destruction of alveoli).

Figure 2 in appendix 10 shows dynamics of elevation of lung wet-to-dry ratio and accumulation of protein in BAL fluid in mice exposed to hyperoxia.

Figure 3 in appendix 10 shows that leukocytes accumulate in the lungs after exposure to hyperoxia and that the kinetics is faster than that of development of the injury.

Figure 4 in appendix 10 shows that level of surface adhesion molecules (CAMs and P-selectin) on the endothelial luminal surface in the lungs increases after exposure mice to hyperoxia.

Figure 5 in appendix 10 shows that leukocytes transmigrate into alveolar space in the lungs of hyperoxia-exposed mice and that blocking of PECAM-1 attenuates this process.

Figure 6 in appendix 10 shows that blocking of CAMs in the lungs by injecting inhibiting antibodies does not attenuate lung injury in hyperoxia model in mice.

Figure 7 in appendix 10 shows that genetic knock-out of CAMs in mice does attenuate lung injury in hyperoxia model.

Figure 8 in appendix 10 shows that depletion of circulating neutrophils does not prevent lung injury in hyperoxia model.

Figure 3 in appendix 8 shows that high-affinity anti-TM/GOX conjugates cause more severe pulmonary oxidative stress and injury than low-affinity counterpart.

Figure 4 in appendix 8 shows that exposure of anti-TM/GOX injected mice to hyperoxia augments lipid peroxidation edema, elevation of BAL protein and tissue injury in lungs of mice injected with anti-TM/GOX.

Figure 5 in appendix 8 shows that level of thrombomodulin expression and pulmonary targeting of anti-TM/GOX are proportional and that both parameters are decreased in mice exposed to hyperoxia.

Figure 6 in appendix 8 shows that normal mice different by gender and age have different natural level of thrombomodulin in their lungs and that this natural variability explains their differential susceptibility to anti-TM/GOX injury.

Table 3. Elevated levels of 8-epi isoprostane (determined by HPLC/MS) and MDA (umol/lung, MDA-586 assay, OxisResearch) in lung homogenates show lipid peroxidation. Protein carbonyls (nm/mg protein) show protein oxidation and myeloperoxidase (MPO) shows neutrophil sequestration. Elevated levels of protein and WBC in the broncho-alveolar lavage (BAL) show edema and white blood cell (WBC) transmigration. Reduced plasma levels of gelsolin, a marker of acute lung injury, show the severity of cellular destruction (Christofidou, 2002).

Parameter	Control	Anti-TM/GOX 30 ug	Anti-TM/GOX 60 ug	48 h 100% O ₂	72 h 100% O ₂
8-epi isoprostane	5.18±0.8	7.21±1.64	19.71±7.22	ND	ND
MDA	4.73±0.42	5.94±0.39	7.84±1.12	6.58±0.03	15.50±0.193
Protein carbonyls	7.5±1.6	ND	ND	15.2±1.8	29.5 (n=1)
MPO	0.23±0.03	0.64±0.04	1.96±0.22	0.56±0.04	0.57±0.09
Lung Wet/Dry ratio	4.23 ±0.1	6.04±0.18	6.67±0.18	4.76±0.06	5.46±0.11
BAL protein	0.14±0.07	0.79±0.71	4.57±1.04	0.928±0.214	4.29±0.54
BAL WBC	58,476±8,901	ND	133,500±45,384	56,838±4,372	72,645±7,958
Plasma gelsolin	373±19	ND	109±18	295±20	110±45

Table 3: Quantitative characterization of oxidant lung injury induced by different doses of anti-TM/GOX or hyperoxia. The data shown as M±SD, n=3-5 (ND: not determined).

Specific Aim 3. Determine protection by anti-CAM/AOE targeted to endothelium in models of pulmonary oxidative stress. In addition to H₂O₂, the main injurious agent in anti-TM/GOX model, diverse Reactive Oxygen Species (ROS) including superoxide anion (superoxide) play a key role in initiation of the cascades of pro-oxidative reactions in the lung tissue in ALI. Thus, both coordinated delivery of diverse AOE and adequate modeling (in the first tier, in cell culture) are needed to develop detoxification of ROS and oxidants and thus achieve profound protective effects. Accordingly, we used diverse models to study CAM targeting and effects of H₂O₂-degrading enzyme catalase, SOD (to detoxify superoxide), and a tandem SOD/catalase, which might permit very effective, coordinated detoxification of both superoxide and H₂O₂ into water, without excessive formation of harmful intermediates.

B.3.1. Protective effects of anti-CAM/AOE conjugates *in vitro*. In the context of ALI and many other pathological conditions, both extracellular (e.g., release by activated leukocytes) and intracellular ROS (i.e., produced by endothelial cells themselves) may induce and propagate vascular oxidative stress in the lungs. It is practically impossible to study intracellular vs extracellular oxidative stress in animal models. In order to test detoxification of intracellular and extracellular endothelial ROS, we studied effects of anti-CAM/AOE in cell cultures.

In vitro characteristics of anti-CAM/catalase, anti-CAM/SOD and anti-CAM/SOD-catalase tandem conjugates. Using both streptavidin and SATA/SMCC cross-linkers we produce anti-CAM/SOD conjugates of optimal size (~250 nm) and documented that: i) the resultant conjugates retain high activity of both SOD and catalase; ii) anti-CAM conjugated SOD and SOD/catalase, but not IgG-conjugated counterparts, bind specifically to endothelial cells in cultures and to REN cells stably transfected with PECAM-1.

Cell culture models of endothelial oxidative stress, apoptosis and lipid peroxidation for testing of effects of anti-CAM/AOE conjugates. We established model of endothelial oxidative stress induced by: i) extracellular ROS produced by xanthine oxidase (XO, which generates both O₂⁻ and H₂O₂ from hypoxanthine and xanthine); and, ii) intracellular ROS, produced by paraquat (which generates O₂⁻ that produces H₂O₂). We characterized cellular viability in these models (using fluorescent labeling of dead and alive cells) and parameters of necrosis (using assay of release of 51Cr and LDH) and apoptosis (using analysis markers of apoptosis, namely conversion of procaspase-3, PARP and ROCK I in treated cells using Western blotting). Both paraquate and XO/X caused cellular toxicity, manifested by necrosis detectable within a few hours after exposure to high doses of insults or apoptosis detectable 20 hours after exposure to the moderate doses. In addition, we established model of ischemic oxidative stress in flow-adapted EC, arguably the most (patho)-physiologically relevant model of vascular oxidative stress initiated by ROS generated by EC. In this model, flow-adapted EC grown in hollow fibers cartridges are challenged with an abrupt cessation of perfusion, leading to activation of ROS-generating enzymatic system(s) in endothelial cells, NDAPH-oxidase, leading to oxidative stress.

Anti-CAM/SOD-catalase tandem affords more effective protection against endothelial necrosis and apoptosis caused by extracellular ROS than anti-CAM/SOD. Treatment of EC with anti-CAM/SOD, but not free SOD, provided significant protection against XO/X induced necrosis. The anti-CAM/SOD protection showed bell-shaped dose dependence curve: the maximal protection (~50%) was reached at a dose of 2.5 µg of SOD per well, while further increasing of SOD targeting resulted in lower protection suggesting that excess of SOD may damage cells, due to overproduction of H₂O₂. Indeed, anti-CAM/SOD/catalase tandem conjugate afforded complete protection against necrotic cell death induced by a large dose of XO. Further, anti-PECAM/SOD/catalase attenuated apoptotic degradation of ROCK-1 in a dose dependent manner, confirming effective antioxidant protection by the tandem AOE conjugate. Therefore, combined detoxification of both superoxide and H₂O₂ attained by SOD and catalase targeted to endothelial cell adhesion molecule affords protection against both necrotic and apoptotic pathways of ROS toxicity.

Anti-CAM/SOD-catalase tandem affords more effective protection against endothelial toxicity caused by intracellular ROS than anti-CAM/SOD. Similarly to results obtained in XO/X model, anti-CAM/SOD conjugate, but not unconjugated SOD, afforded highly significant, ~50% protection against paraquat-induced toxicity even at very low doses (0.5-1.0 µg/well). Protection was highly specific, since treatment of CAM-negative REN cells with the conjugate produced no protection against paraquat. Similarly to the model of extracellular ROS attack induced by XO/X, anti-PECAM/SOD conjugates showed bell-shaped protection curve in paraquat-induced intracellular oxidative stress. The maximal protection (~50%) was reached at a dose of 1 µg of SOD per well, while increase of SOD dose above 10 µg resulted in less effective protection, likely due to overproduction of H₂O₂ from superoxide. In support of this hypothesis, tandem anti-CAM/SOD-catalase conjugate afforded more complete (>90%) protection against paraquat, as we observed in XO/X model.

Anti-CAM/SOD-catalase eliminates ROS produced by EC in response to ischemia. Encouraged by protection by anti-CAM/SOD/catalase conjugate in static culture of EC exposed to chemically generated external and internal ROS, we tested its effect in the model of EC ischemia. To detect ROS, we preloaded cells with DCF, a probe that fluoresces in response to ROS. In this series, perfusion of a mixture of non-conjugated SOD and catalase afforded only partial reduction of DCF fluorescence, while anti-PECAM/SOD/catalase conjugate abolished fluorescence induced by ischemia, indicating effective elimination of endothelial ROS.

Comprehensive description of the results:

Figure 4B in appendix 1 shows that fluorescent microscopy reveals that anti-CAM/catalase, but not IgG/catalase conjugate specifically binds to endothelial cells.

Figure 5 in appendix 1 shows that anti-CAM/catalase conjugate enhances H₂O₂-degrading capacity of the endothelial cells (panel A) and effectively protects endothelial cells from cytotoxic effect of hydrogen peroxide (revealed by release of ⁵¹Cr, panel B, and by preserved cellular morphology, panel C).

Figure 1 in appendix 11 shows synthesis, enzymatic activity, size and specific binding to endothelial cells in culture of anti-PECAM/SOD conjugate.

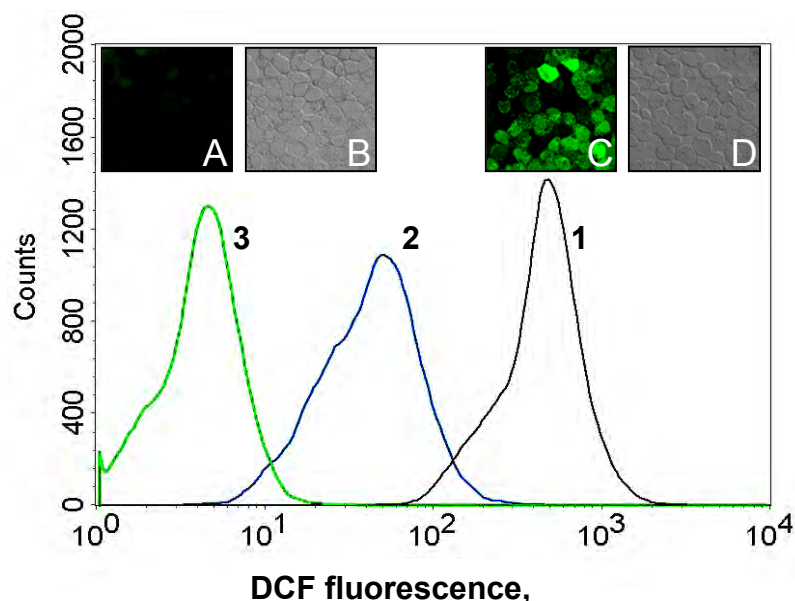
Figure 2 in appendix 11 shows dose dependence of pro-apoptotic effect of xanthine-xanthine oxidase (XO/X) in endothelial cell culture and dose-dependent protective effect against XO/X achieved in this model by anti-PECAM/SOD, but not unconjugated SOD.

Figure 3 in appendix 11 shows that exposure of endothelial cells to high dose of XO/X causes their necrosis and that anti-PECAM/SOD partially, and tandem anti-PECAM/SOD/catalase conjugate completely protects cells against XO/X toxicity.

Figure 4 in appendix 11 shows that paraquat (PQ) causes dose-dependent cell toxicity and that specific PECAM-directed targeting of anti-PECAM/SOD completely protects against PQ toxicity.

Figure 1 below shows protective effect of anti-CAM/SOD and tandem anti-CAM/SOD/catalase conjugates against oxidative stress in a more physiological model of ischemia/reperfusion injury in flow-adapted endothelial cells grown in hollow fibers cartridges. In order to test whether AOE immunotargeting intercepts endogenous ROS produced by endothelial cells, we utilized a model of flow-adapted HUVEC, which produce O_2^- or/and H_2O_2 via NADPH-oxidase dependent pathway in response to ischemia. To detect ROS, we preloaded cells with DCF, a probe that fluoresces in response to over-production of ROS. Perfusion of a mixture of non-conjugated SOD and catalase afforded only partial reduction of DCF fluorescence, while anti-PECAM/SOD/catalase conjugate abolished fluorescence induced by ischemia.

Fig.2. Protection of endothelial cells against ischemia-mediated ROS generation. Histogram shows FACS analysis of DCF fluorescence in control ischemic cells (trace 1) and in cells treated with mixture of free catalase, SOD and anti-PECAM (trace 2) and equal amount of PECAM/SOD/catalase conjugate (trace 3). Inset, fluorescent microscopy (A and C) and phase contrast images (B and D) of untreated (A-B) and conjugate-treated cells (C-D).



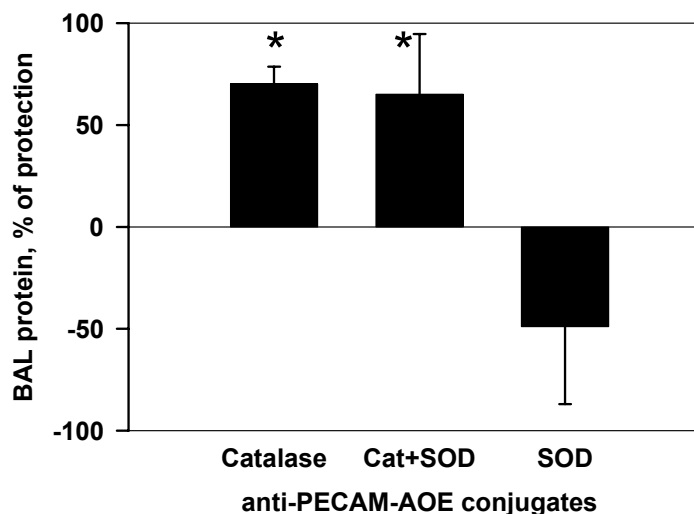
B.3.2. Protective effects of anti-CAM/AOE conjugates in animal models of pulmonary oxidative stress. Studies in the Aim 1 showed that anti-CAM conjugated catalase, SOD and SOD-catalase tandem specifically accumulate in mouse and rat lungs after IV injection. Based on this and the highly encouraging

in vitro data accumulated in the previous years of the grant, in the fifth year we tested protective effects of anti-PECAM/SOD, anti-PECAM/catalase and anti-PECAM/SOD-catalase in several animal models of pulmonary oxidative stress: i) anti-TM/GOX; ii) oxidative vasoconstriction, and iii) lung ischemia/reperfusion. The results obtained in the ischemia model will be described in the Specific Aim 5, since we have studied effects of both antioxidant and anti-thrombotic targeting in this most complex and clinically relevant model.

Pulmonary targeting of anti-CAM/catalase, but not anti-CAM/SOD is protective against pulmonary oxidative stress induced by anti-TM/GOX. Using the model described in the Aim 2, we compared protective effects of anti-PECAM/catalase, anti-PECAM/SOD and anti-PECAM/SOD-catalase against mouse lung injury induced by generation of H₂O₂ in endothelial cells. We have found that anti-PECAM/SOD provided no protective effect in this model and that anti-PECAM/SOD-catalase tandem conjugate provided no better protection than anti-PECAM/catalase. This result fits well with results described in the Section B2 indicating that H₂O₂ is the prime injurious factor in this model, while subsequent infiltration of WBC into the lungs (which may serve as secondary source of superoxide) does not contribute to the tissue injury and rather reflects physiological response to GOX-inflicted damage.

Comprehensive description of results:

Figure 3. Anti-PECAM/catalase, and anti-PECAM/SOD-catalase tandem conjugate, but not anti-PECAM/SOD conjugate, protected against pulmonary edema caused by anti-TM/GOX.

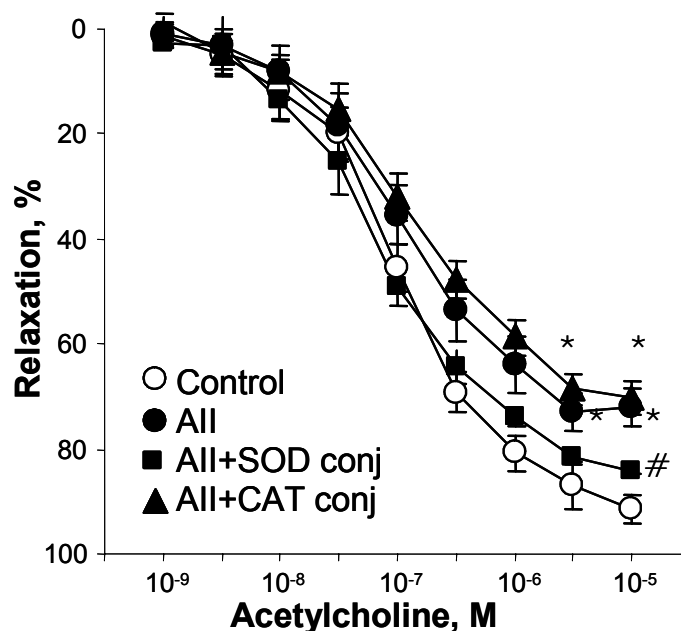


Targeting of anti-CAM/SOD, but not anti-PECAM/catalase is protective against superoxide-induced vasoconstriction. In order to test effect of SOD delivery in a pathological condition associated with endothelial production of superoxide, we used a model in which a miniature pump implanted in a mouse infuses Ang II, a vasoactive peptide that enhances endothelial production of superoxide that inactivates NO and thus causes pathological vasoconstriction.

Injection of anti-PECAM/SOD, but not non-targeted SOD completely normalized vascular tone in these animals, while anti-PECAM/catalase had no protective effect. This result indicates that targeting SOD is beneficial in a setting in which superoxide anion is a primary pathological mediator and validates anti-PECAM/SOD activity in vivo.

Comprehensive description of results:

Figure 4. Targeting of anti-PECAM/SOD, but not anti-PECAM/catalase protects against vasoconstriction caused by infusion of Ang II in mice: significantly higher doses of acetylcholine are needed to attain relaxation of the vessels obtained from Ang II-infused mice treated with catalase conjugate vs SOD conjugate.



B.4. Specific Aim 4. Determine fibrinolysis by anti-CAM/PA targeted to endothelial surface. In this Aim, we have designed a series of anti-CAM PA conjugates and fusion proteins and characterized their effects in animal models of thrombosis. In particular, we: i) compared fibrinolytic activity of diverse PA in mice to select optimal cargoes; ii) designed chemical anti-CAM/tPA conjugates; iii) synthesized and tested anti-CAM scFv/uPA fusion pro-drugs activated by either plasmin or thrombin. Our results indicate that the latter formulation is optimal for subsequent industrial development and therapeutic utility.

B.4.1. Testing of PA drugs, carriers and anti-CAM/PA conjugates. In the first phase of the study, utilizing currently available PA formulations, we defined forms of PA suitable for endothelial targeting and demonstrated proof of principle for such a targeting using chemical conjugation of PA to anti-CAM [10].

Selection of optimal plasminogen activators. In order to select best drugs for targeting to EC, we compared wild-type tPA, Retavase, Tenektase and urokinase. Activity of these PA was tested using chromogenic substrate and dissolution of fibrin clots containing plasminogen. In this model, four PA produced equally effective fibrinolysis with similar kinetics. In human plasma clots, however, lytic activity was in the order Tenektase>tPA>>Retavase>urokinase, which reflected their susceptibility to plasma inhibitors. By injecting these activators in mice and testing subsequent fibrinolytic activity in the blood we

found that tenektase shows highest constitutive activity, while urokinase is a pro-drug exerting no appreciable activity in blood under normal conditions.

Pulmonary targeting and fibrinolytic activity of anti-ICAM/PA complex in rats and mice. To obtain a proof of principle for facilitated pulmonary fibrinolysis by anti-CAM/PA targeting, we synthesized large (~1 μ m), poorly internalizable anti-ICAM/tPA and IgG/tPA conjugates. Animal studies that: i) anti-ICAM/tPA, but not IgG/tPA accumulates in the lungs of rats and mice after IV injection (isotope tracing); ii) retains its enzymatic activity in the pulmonary vascular lumen; and, iii) facilitates subsequent dissolution of fibrin emboli in the lungs. Therefore, anti-CAM/PA accumulates in the lungs, resides in enzymatically active form on the luminal endothelial surface, and thereby markedly facilitates fibrinolysis in the pulmonary vasculature [10].

B.4.2. Design and testing of recombinant pro-drug urokinase fused with anti-CAM single-chain antigen-binding fragment, anti-PECAM scFv. Studies with chemical conjugates provided proof of principle, but these formulations are sub-optimal for industrial development and human use. To solve this problem, we designed genetically engineered fusion proteins combining a specific target-binding site of the affinity moiety (single chain antigen-binding fragment, or anti-PECAM scFv) and a pro-drug urokinase (uPA).

Anti-CAM scFv/uPA provides prophylactic fibrinolysis in the mouse lungs. We synthesized a plasmin-activated pro-drug scFv/uPA mutant lacking unnecessary uPA domains that could cause side effects. Series of in vitro and in vivo studies showed that this fusion protein: i) retains functional activity; ii) binds to EC and resides on EC surface in vitro and after injection in mice; iii) accumulates in the lungs after IV injection in mice, but not in mutant mice lacking CAM determinant; and, iv) markedly facilitates subsequent dissolution of pulmonary emboli [13].

Synthesis and successful testing of advanced anti-CAM scFv/uPA-T activated by thrombin. To further improve the specificity, safety and durability of this novel type of pro-drug, we mutated scFv/uPA and converted plasmin-activated site into thrombin-activated site. This fusion: i) Binds to CAM and EC; ii) Is not sensitive to plasmin, but gets activated and released from CAM anchor by thrombin; iii) Is resistant to inhibitors until thrombin activation; iv) does not cause activation of plasminogen and consumption of fibrinogen in normal mice (in contrast to uPA, which caused this side effect); v) accumulates in the lungs after IV injection in mice; and, vi) causes more potent and durable lysis of subsequent pulmonary clots formed by intravascular production of thrombin than plasmin-activated scFv/uPA prototype. These results indicate that thrombin activated scFv/uPA-T is a preferable agent to providing safe, specific and durable prophylactic thrombolysis in the pulmonary vasculature.

Comprehensive description of results:

Figure 8 in appendix 6 shows that anti-ICAM/tPA conjugate specifically accumulates in the pulmonary vasculature after IV injection in rats.

Figure 8 in appendix 6 shows that anti-ICAM/tPA conjugate is functionally active in the pulmonary vascular lumen and augments dissolution of pulmonary thrombi in perfused rat lungs.

Figure 1 in appendix 12 shows that a fusion protein anti-PECAM scFv/uPA is synthesized with correct predicted size of ~60 kD.

Figure 2 in appendix 12 shows that this fusion protein specifically binds to endothelial cells in cell culture, as revealed by fluorescent microscopy and ELISA.

Figure 3 in appendix 12 shows that this fusion protein possesses urokinase enzymatic activity, gets activated by plasmin, causes dissolution of fibrin clot in vitro and displays enzymatic activity after binding to endothelial cells.

Figure 4 in appendix 12 shows that this fusion protein accumulates specifically in mouse lungs after IV injection and retains in the pulmonary vasculature for at least several hours.

Figure 6 in appendix 12 shows that this fusion protein causes significantly more profound augmentation of dissolution of fibrin clots in mouse lungs than non-targeted urokinase.

Figure 1 in the 3rd annual report attachment shows that a new, thrombin-activated anti-PECAM scFv/uPA-T fusion protein is synthesized with correct predicted size (~60 kD), binds specifically to immobilized PECAM and is sensitive to thrombin cleavage.

Figure 2 in the 3rd annual report attachment shows that scFv/uPA-T is enzymatically activated by thrombin, but not plasmin and thus dissolves fibrin clot.

Figure 3 in the 3rd annual report attachment shows that native scFv/uPA-T is resistant to plasminogen activator inhibitor, PAI-1.

Figure 4 in the 3rd annual report attachment shows that scFv/uPA-T accumulates in the mouse lungs after IV injection and is retained in the pulmonary vasculature for many hours

Figure 5 in the 3rd annual report attachment shows that scFv/uPA-T causes no systemic activation of plasminogen and depletion of fibrinogen in vitro and in animals.

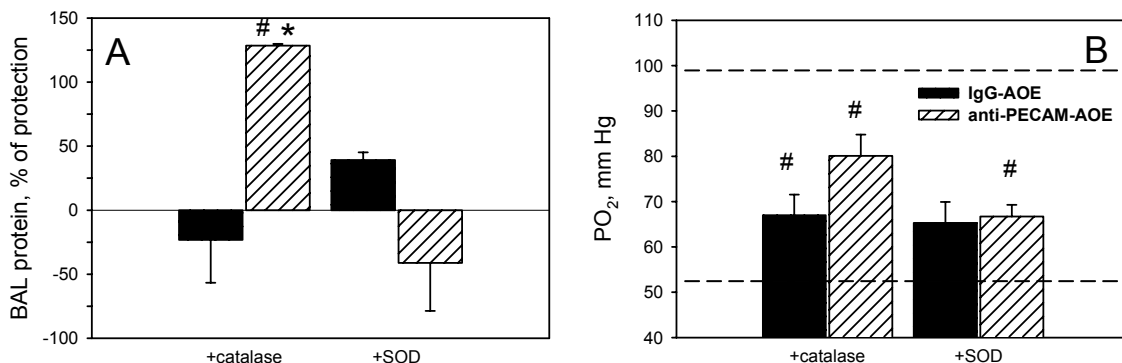
Figure 6 in the 3rd annual report attachment shows that scFv/uPA-T causes more effective and prolonged dissolution of blood clots in the pulmonary vasculature of mice injected with tissue factor than both non-targeted uPA and plasmin-activated scFv/uPA.

B.5. Specific Aim 5. Evaluate the therapeutic potential of anti-thrombotic and anti-oxidant drugs immunotargeting to CAM in animal models.

In the last Specific Aim, pursued mostly in the fifth year of the grant, we estimated potential utility of targeting AOE and PA to the pulmonary endothelium. For this purpose, we used a model of unilateral in situ ischemia-reperfusion (1 hour and 1 hour) in the ventilated anesthetized mice. This procedure inflicts severe tissue injury characterized by both oxidative and thrombotic stress in the pulmonary vasculature, manifested by severe drop in blood oxygenation level (to 50% of control level) and accumulation of protein in the BAL, reflecting oxidative injury, as well as accumulation of fibrin, reflecting thrombosis. In the recent series we

have tested effects of anti-CAM/AOE conjugates and anti-CAM scFv/uPA-T in this model.

Pulmonary targeting of anti-CAM/catalase, but not anti-CAM/SOD is protective against pulmonary oxidative stress induced by ischemia-reperfusion. Anti-TM/GOX and Ang-II models used in the Aim 3 cause vascular oxidative stress induced by predominantly H₂O₂ or superoxide, respectively. Thus they provided good model systems to validate targeting of catalase vs SOD, respectively. In most of clinical conditions associated with vascular oxidative stress, however, both superoxide and H₂O₂ are produced in oxidative stress. In order to address role of detoxification of superoxide anion and H₂O₂ in a more physiologically relevant model, we used lung ischemia-reperfusion model in mice. Pre-treatment (IV injection 15 min prior to ischemia) of anti-PECAM/catalase, but not anti-PECAM/SOD, provided significant protection against ischemia/reperfusion injury according to both these parameters. Interestingly, anti-PECAM/SOD-catalase conjugate provided no better effect than anti-PECAM/catalase alone. This interesting outcome indicates that in this physiological model of vascular oxidative stress, H₂O₂ apparently is the main injurious agent. However, this outcome also indicates that in general targeting strategy for delivery of AOE to EC works in vivo and can be tested in experimental models of human oxidative stress including ALI/ARDS.



Comprehensive description of results:

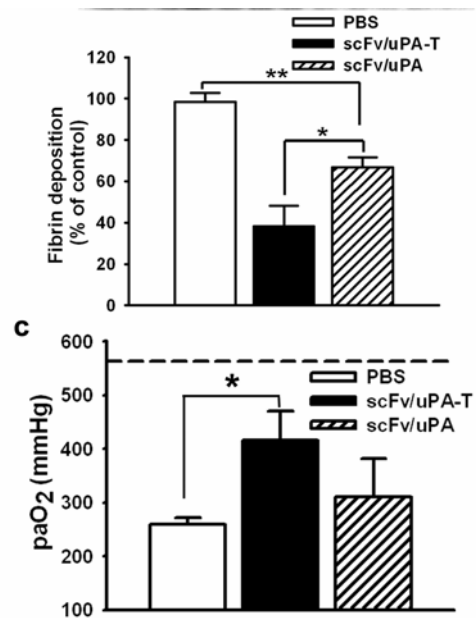
Figure 5 shows that anti-PECAM/catalase, but not anti-PECAM/SOD, significantly protects against lung ischemia-reperfusion. Either IgG conjugate (closed bars) or anti-PECAM conjugate (hatched bars) of indicated enzymes were injected in mice prior to I/R. Panel A shows protection against lung edema, panel B shows preservation of pulmonary function of blood oxygenation.

Pulmonary targeting of anti-CAM scFv/uPA-T is protective against pulmonary thrombosis induced by ischemia-reperfusion. In the last series of experiments, we injected mice with thrombin-activated anti-CAM scFv/uPA-T vs plasmin activated prototype 15 min prior to unilateral lung ischemia-reperfusion. Data of the pilot series indicate that thrombin-activated fusion protein prevents fibrin deposition in the lungs and markedly improves blood oxygenation. Therefore, anchoring of fibrinolytic pro-drugs to the pulmonary vascular lumen can be used

(most likely, in concert with targeting of anti-CAM/AOE) for protection against vascular thrombotic and oxidative stress.

Comprehensive description of results:

Figure 6 shows that thrombin-activated targeted anti-PECAM scFv/uPA-T affords significantly more effective protection against thrombosis (fibrin deposition, upper panel) and hypoxia (lower panel) caused by lung ischemia-reperfusion in mice in situ.



C. Key Research Accomplishments.

- Design, structure, size, functional activities and stability of anti-CAM/AOE conjugates have been fully characterized, optimized and are suitable subsequent development and pre-clinical and clinical testing;
- The mechanisms of endothelial internalization, traffic, destination and metabolism of anti-CAM/conjugates are characterized;
- Main parameters of these processes (amount of delivered conjugates, rate of internalization, lysosomal traffic and duration of the AOE in the cells) are suitable of anti-CAM/AOE utility for protection against acute oxidative stress;
- Means for pharmacological control of intracellular traffic and metabolism of anti-CAM/AOE have been defined and shown to provide marked prolongation of protective effects of anti-CAM/AOE;
- Internalizable stable anti-CAM conjugates accumulating within endothelial cells do not affect their viability, division and vesicular traffic;
- Geometry of the conjugates (size and shape) controls their uptake by the EC and targeting to the pulmonary EC;
- Quantitative analysis of CAM-directed targeting of the AOE conjugates and PA fusion proteins to the pulmonary endothelium is characterized in perfused lungs, in intact animals and in the animal models of lung pathology;
- Models of endothelial oxidative stress (necrotic and apoptotic versions) induced by diverse extracellular or intracellular ROS including superoxide anion and H₂O₂ in endothelial cells have been established;
- Protective activity of anti-CAM/SOD and tandem anti-CAM/SOD/catalase conjugates has been demonstrated and characterized in these models, with tandem conjugate producing greater protection in all tested models, including ischemia-induced ROS generation in flow adapted EC;
- Multifaceted parameters characterizing pulmonary injury in mouse models of acute lung injury induced by diverse formulations of anti-TM/GOX, hyperoxia and combined anti-TM/GOX and hyperoxia have been characterized, including dynamics of expression CAM and WBC migration.
- Optimal plasminogen activators (pro-drug urokinase) and targeting means (anti-CAM scFv) have been defined, recombinant fusion constructs combining anti-CAM scFv and uPA has been designed, synthesized and characterized (including verification of the proper size, folding, antigen-binding and enzymatic activities);
- Anti-CAM scFv/uPA pro-drug activated by plasmin has been tested and showed binding to EC in cell cultures and in intact animals, accumulation in the lungs and facilitation of dissolution of pulmonary thrombi in mice;
- Thrombin-activated anti-CAM scFv/uPA-T construct, an ideal candidate for industrialization and translation into pre-clinical and clinical studies, have been designed and successfully tested in mouse model of pulmonary thrombosis;
- Anti-PECAM scFv-scuPA-T is a real pro-drug that can be converted into a fully active plasminogen activator by thrombin that cleaves a scuPA moiety of the fusion construct and endows it with high enzymatic and fibrinolytic activity. It is fully resistant to plasmin, which minimizes potential

systemic activation of the drug. Anti-PECAM scFv-scuPA-T construct is an ideal pro-drug for local interventions into vascular thrombosis;

- Protective effects of anti-CAM/AOE and scFv/uPA-T have been tested and showed highly promising protective effects in animal models of pulmonary oxidative stress and thrombosis including highly clinically relevant model of lung ischemia-reperfusion.

D. Reportable Outcomes

We organized this section in a way that permits to maintain confluence of reported publications that naturally overlaps between the grant years, such as "in press" in year 4 vs "published" in year 5. We show *outcomes reported in the previous four years of the grant in Italics*, followed by an extra-space and a list of publications submitted or published within the current funding year.

The total number of publications is:

Full-size papers and chapters – 23;

Published abstracts of scientific conferences and meetings - 32;

Lectures in scientific meetings and research seminars - 31.

D.1. Full-size peer-reviewed papers (* papers in which the PI is senior author):

1. S.Muro, R.Wiewrodt, A.Thomas, L.Koniaris, S.Albelda, **V.Muzykantov*** and M.Koval (2003) A novel endocytic pathway induced by clustering endothelial ICAM-1 or PECAM-1. *J Cell Sci.* 2003;116(Pt 8):1599-1609
2. **V.Muzykantov** (2003) Targeting pulmonary endothelium. in: "Biomedical Aspects of Drug Targeting", **Vladimir Muzykantov** and Vladimir Torchilin, Eds., Kluwer Academic Publishers, Boston-Dodrecht-London, pages 129-148
3. J.C.Murciano, S.Muro, L.Koniaris, M.Christofidou-Solomidou, D.Harshaw, S.Albelda, D.Granger, D.Cines and **V.R.Muzykantov*** (2003) ICAM-directed vascular immunotargeting of anti-thrombotic agents to the endothelial luminal surface. *Blood*, 101:3977-1984
4. S.Muro, X.Cui, C.Gajewski, **V.Muzykantov*** and M.Koval (2003) Slow intracellular trafficking of catalase nanoparticles targeted to ICAM-1 protects endothelial cells from oxidative stress. *Am.J.Physiol., Cell Physiol.*, 285(5):C1339-47.
5. V.Shuvaev, T.Dziubla, R.Wiewrodt, and **V.Muzykantov*** (2004) Streptavidin-biotin cross-linking of therapeutic enzymes with carrier antibodies: nanoconjugates for protection against endothelial oxidative stress. "Methods in Molecular Biology. Bioconjugation Protocols: Strategies and Methods", C.M.Niemeyer, Ed., Humana Press, Totowa, NJ, Chapter 1, pp 3-18
6. S.Muro, **V.Muzykantov*** and J.Murciano (2004) Characterization of endothelial internalization and targeting of antibody-enzyme conjugates in cell cultures and in laboratory animals "Methods in Molecular Biology. Bioconjugation Protocols: Strategies and Methods", C.M.Niemeyer, Ed., Humana Press, Totowa, NJ, Chapter 2, pp 21-36
7. S.Muro, M.Koval and **V.Muzykantov*** (2004) Endothelial endocytic pathways: gates for vascular drug delivery. *Current Vascular Pharmacology*, 2:281-299
8. S.Muro, C.Gayewsky, M.Koval and **V.Muzykantov** (2005) ICAM-1 recycling in endothelial cells: a novel pathway for sustained intracellular delivery and prolonged effects of drugs. *Blood*, 105:650-658
9. S.Muro and **V.Muzykantov*** (2005) Targeting of antioxidant and anti-thrombotic drugs to endothelial cell adhesion molecules. *Current Pharmacological Design*, 11, 2383-2401
10. **V.Muzykantov** (2005) Targeting drugs to pulmonary endothelium. *Expert Opinion on Drug Delivery*, 2:909-926
11. M.Christofidou-Solomidou and **V.Muzykantov*** (2006) Antioxidant Strategies in Respiratory Medicine. *Treatments in Respiratory Medicine*, 5 (1): 47-78

12. **V.Muzykantov*** and S.Danilov (2006) *Delivery of therapeutic and genetic materials to vascular endothelium. "Encyclopedia of the Microvasculature" (D.Sherpo and J.Garcia, Editors), Elsevier Science (USA), Chapter 155, pages 1053-1059*
13. B.Ding, T.Dziubla, V.Shuvaev, S.Muro and **V.Muzykantov*** (2006) *Advanced drug delivery systems that target vascular endothelium. Mol.Interventions, 6(2):98-112*
14. T.Dziubla and **V.Muzykantov** (2006) *Synthetic carriers for vascular delivery of protein therapeutics. Biotechnology and Genetic Engineering Reviews (S.E.Harding, Ed., Lavoisier-Intercept Publishers, Paris), vol 22, chapter 13, pp: 267-298*
15. T.Dziubla, S. Muro, **V.R.Muzykantov** and M. Koval (2007) *Nanoscale Antioxidant Therapeutics. In Oxidative Stress, Disease and Cancer, Keshav K. Singh, Editor, Imperial College Press, London., in press*
16. T.Dziubla and **V.Muzykantov** (2007) *Delivery of nanoparticles to the lungs. "Nanoparticles as drug carriers" (V.Torchilin, Editor), in press*
17. Bi-Sen Ding, C.Gottstein, A.Grunow, A.Kuo, K.Ganguly, S.Albelda, D.Cines and **V.R.Muzykantov*** (2005) *Endothelial targeting of a recombinant construct fusing a PECAM-1 single-chain variable antibody fragment (scFv) with pro-urokinase facilitates prophylactic thrombolysis in the pulmonary vasculature. Blood, 106:4191-4198*
18. S.Muro, E.Schuchman and **V.R.Muzykantov** (2006) *Lysosomal enzyme delivery by ICAM-1 targeted nanocarriers bypassing glycosylation and clathrin-mediated endocytosis. Mol.Therapy, 13:135-141*

19. S.Muro, M.Matescu, C.Gayewsky, **V.Muzykantov*** and M.Koval (2006) *Control of intracellular trafficking of ICAM-1 targeted nanocarriers by endothelial Na/N exchanger (NHE) proteins. Am.J.Physiol. Lung Cell Mol.Physiol., 290:L809-817 (Editorial Highlight)*
20. S.Muro, T.Dziubla, W.Qui, J.Leferovich, X.Cui, E.Berk and **V.R.Muzykantov*** (2006) *Endothelial targeting of high-affinity multivalent polymer nanocarriers directed to ICAM-1. J.Pharm.Exp.Therap., 317:1161-1169*
21. S.Perkowski, A.Scherpereel, J-C.Murciano, E.Arguiri, C.Solomides, S.Albelda, **V.R.Muzykantov** and M.Christofidou-Solomidou (2006) *Dissociation between alveolar transmigration of neutrophils and lung injury in hyperoxia. Am.J.Physiol. Lung Cell Mol.Physiol., 291(5):L1050-8*
22. V.Shuvaev, M.Christofidou-Solomidou, A.Scherpereel, E. Simone, E.Arguiri, S.Tliba, J.Pick, S.Kennel, S.Albelda and **V.R.Muzykantov*** (2007) *Factors modulating the delivery and effect of enzymatic cargo conjugated with antibodies targeted to the pulmonary endothelium J.Controlled Release, 118:235-244*
23. V.Shuvaev, S.Tliba, M.Nakada, S.Albelda and **V.R.Muzykantov*** (2007) *PECAM-directed endothelial targeting of SOD alleviates oxidative stress caused by either extracellular or intracellular superoxide. J.Pharm.Exp.Therap., 323, in press*

D.2. Published presentations at international and national scientific conferences.

1. S.Muro, A.Thomas, **V.Muzykantov**, M.Koval (2002) PKC-mediated endocytosis of conjugates targeted to ICAM-1. Abstracts of Experimental Biology 2002 Meeting, New Orleans, LA, April 20-24, 2002 (A.382.12) FASEB J., 2002, 16(4), p A439
2. A.Scherpereel, J.C.Murciano, R.Wiewrodt, S.Kennel, **V.Muzykantov**, M.Christofidou-Solomidou (2002) Glucose oxidase vascular immunotargeting to pulmonary endothelium enhances luminal expression of ICAM-1 and P-selectin in the lung. Abstracts of Experimental Biology 2002 Meeting, New Orleans, LA, April 20-24, 2002 (A.382.13) FASEB J., 2002, 16(4), p A439
3. S. Muro, M.Koval, A.Thomas and **V.Muzykantov** (2002) Affinity carriers targeted ICAM-1: trafficking into endothelial cells. Abstracts of American Thoracic Society International Conference, Atlanta, GA, May 17-22, 2002; Am.J.Resp.Crit.Care Med., 2002; 165 (4):A101
4. A.Thomas, T.Sweitzer, R.Wiewrodt, S.Muro, M.Koval and **V.Muzykantov** (2002) Size-dependent intracellular targeting of immunoconjugates directed against endothelial surface adhesion molecules. Abstracts of XIV World Congress of Pharmacology, San Francisco, CA July 7-12, 2002; Pharmacologist, 2002, 44, #2 (suppl. 1), 61.2
5. S.Muro, T.Sweitzer, R.Wiewrodt, L.Koniaris, A.Thomas, S.Albelda, M.Koval and **V.Muzykantov** (2002) Kinetics of intracellular immunotargeting to endothelium via adhesion molecules. Ibid, 61.3
6. **V.Muzykantov** (2002) Catalase immunotargeting: protection against oxidative stress in the pulmonary vasculature. Abstr. Int.Symposium "Reactive Oxygen and Nitrogen Species", St.Petersburg-Kizhi, Russia, 8-12 July, 2002, Palma Press, Moscow, p.58
7. M.Christofidou, S.Muro, J.Murciano, M.Barry, A.Thomas, V.Shuvaev, S.Albelda, D.Cines and **V.R.Muzykantov** (2003) Targeting endothelial surface adhesion molecules. Abstr. Symposium of Vector Targeting, Cold Spring Harbor, NY, March 20-22, 2003, page 19)
8. M.Christofidou, A.Scherpereel, A.Boyen, E.Arguiri, V.Shuvaev, S.Kennel and **V.Muzykantov** (2003) Hyperoxia potentiates oxidative injury in murine lungs induced by glucose oxidase targeted to thrombomodulin. Abstr., Experimental Biology Meeting, San Diego, CA, April 11-15, 2003; FASEB J., 2003; 17(4) part I, page A247
9. S.Muro, X.Cui, C.Gajewski, M.Koval, **V.Muzykantov** (2003) Pharmacological Modulation of Intracellular Trafficking and Lysosomal Degradation Prolongs the Anti-oxidant Effect of Catalase Conjugates Delivered into Endothelial Cells via ICAM-1. Abstr. 11th Intern.Symp. Recent Advances in Drug Delivery Systems, Salt Lake City, UT, March 3-6, 2003 (#014)
10. M.Christofidou-Solomidou, B.Kozower, A.Scherpereel, T.Sweitzer, S.Muro, R.Wiewrodt, V.Shuvaev, A.Thomas, M.Koval, A.Patterson, S.Albelda and **V.Muzykantov**. Targeting of antioxidants to adhesion molecules (2003) Inflammation Research, 52, Suppl.2, p.S81 (Abstracts of 6th World Congress on Inflammation, Vancouver, Canada, August 2-6, 2003)
11. E.Berk, **V.Muzykantov** and S.Muro (2003) Binding and uptake of anti-ICAM-1 coated nanoparticles by flow-adapted endothelial cells. Abstr. 9th Annual Respiration Research Retreat of the University of Pennsylvania, Sugarloaf Conference Center, Philadelphia, PA, June 20, 2003, #9

12. M.Christofidou-Solomidou, A.Scherpereel, A.Bohen, E.Arguiri, V.Shuvaev, S.Kennel, **V.Muzykantov** (2003) Hyperoxia potentiates oxidative injury in murine lungs induced by glucose oxidase targeted to thrombomodulin. *Ibid*, #16
13. S.Muro, C.Gajewsky, M.Koval, **V.Muzykantov** (2003) Slow intracellular degradation of ICAM-1 or PECAM-1 targeted catalase nanoparticles protects endothelial cells from oxidative stress. *Ibid*, #49
14. V.Shuvaev, S.Tliba, T.Dziubla, **V.Muzykantov** (2003) Combined immunoconjugate delivery of CuZnSOD and catalase to human endothelial cells and their protection against oxidative stress. *Ibid*, #55
15. M.Christofidou-Solomidou, V.Shuvaev, A.Scherpereel, E.Arguiri, S.Tliba and **V.Muzykantov**. (2004) Vascular immunotargeting of catalase as SOD using an anti-PECAM carrier ameliorates oxidative lung injury in double hit mouse model. *Abstr. International ATS Conference, May 21-26, 2004, Orlando FL, Am.J.Resp.Ctric.Care Med.*, 169, page A874
16. V.Shuvaev, S.Tliba, K.Laude, D.Harrison, V.Muzykantov (2004) PECAM-targeted delivery of superoxide dismutase to endothelium protects against xanthine oxidase induced oxidant stress and Ang II-induced hypertension. *Abstr. 10th Annual Respiration Research Retreat of the University of Pennsylvania, Sugarloaf Conference Center, Philadelphia, PA, June 11, 2004, p.41*
17. S.Muro, C.Gajewski, M.Koval, V.Muzykantov (2004) Sustained endothelial delivery of catalase via ICAM-1 recycling pathway. *Ibid*, p.33
18. K.Laude, V.Shuvaev, A.Bikineyeva, S.Dikalov, **V.Muzykantov**, D.Harrison. Endothelial immunotargeting of superoxide dismutase corrects endothelial dysfunction in angiotensin II-induced hypertension. *Hypertension*, 2004, vol. 44, #4 *Abstr. 58th AHA Conference on High Blood Pressure Research, Chicago, IL, October 9-12, 2004, page 555*
19. S.Muro and V.Muzykantov (2004) Sustained drug delivery into endothelium via ICAM-1. *Abstr. of AHA Grover Conference on Pulmonary Circulation, Lost Valley Ranch, CO, September 1-12, 2004, Abstr.#12*
20. S.Muro, C.Gajewsky, M.Koval and **V.Muzykantov** (2004) Sustained drug delivery into endothelial cells via recycling ICAM-1. *Abstr. of International Symposium on Molecular Design in Drug Development and Discovery, Toronto, Canada July 8-10, 2004, Abst.#9*
21. M.Christofidou-Solomidou, V.Shuvaev, A.Scherpereel, E.Arguiri, S.Tliba and **V.Muzykantov**. (2004) Vascular immunotargeting of catalase as SOD using an anti-PECAM carrier ameliorates oxidative lung injury in double hit mouse model. *Abstr. International ATS Conference, May 21-26, 2004, Orlando FL, Am.J.Resp.Ctric.Care Med.*, 169, page A874
22. S.Muro, J.C.Murciano and **V.R.Muzykantov** (2004) Targeting of drugs to cell adhesion molecules for treatment of acute lung injury. *Proceedings of the DOD PRMRP Military Health Research Forum, San Juan, Puerto Rico, 25-28 April, 2004, Page 16*
23. S.Muro, C.Gajewsky, M.Koval and **V.Muzykantov** (2004) Sustained endothelial delivery of catalase via ICAM-1 recycling pathway. *Ibid.*, p 23
24. B.Ding, C.Gottstein, D.Cines, A.Kuo, S.Zaitsev, T.Krasik, K.Ganguly, V.Muzykantov (2004) A genetically engineered anti-PECAM/urokinase fusion construct for targeting fibrinolysis to pulmonary vasculature. *Abstr. of 21st*

- Annual Pharmacological Sciences Student Symposium of University of Pennsylvania, Gregg Center, Bryn Mawr, PA, 8 October 2004, p.17*
25. C.Gajewsky, S.Muro, M.Koval and **V.Muzykantov** (2004) Sustained endothelial delivery of catalase via ICAM-1 recycling pathway. *Ibid.*, p 18
 26. V.Muzykantov, S.Muro, T.Dziubla, V.Shuvaev (2004) Endothelial adhesion molecules as therapeutic targets. *Abstr. of Institute of Medical Engineering Symposium, University of Pennsylvania, December 2, 2004, p.8*
 27. W.Qui, S.Muro, T.Dziubla, V.Muzykantov (2004) Characterization of surface-adsorbed antibody particle binding to endothelium. *Ibid*, p.70
 28. V.Shuvaev, S.Tliba, K.Laude, D.Harrison, V.Muzykantov (2004) Targeted delivery of SOD via immunoconjugation to endothelium protects against xanthine oxidase-induced oxidant stress and angiotensin II-induced hypertension. *Ibid*, p.71
 29. V.Shuvaev, J.Pack, S.Tliba, D.Dziubla and **V.Muzykantov** (2005) Superoxide dismutase nanoconjugates targeted to endothelium augment antioxidant protection. *Abstr. Exp.Biology Meeting, March 31-April5, San Diego, CA, FASEB J., 2005, 19/5, pA1207*
 30. T.Dziubla, V.Shuvaev, S.Tliba and **V.R.Muzykantov**. Prolonged protection of endothelium from oxidative stress by targeting antioxidant enzyme-loaded polymer nanocarriers. *Proc. 3rd International Symposium on Nanomedicine and Drug Delivery, Baltimore, MD, September 26-27, 2005, page 76*
 31. S.Muro, T.Dziubla, W.Wui, J.Leferovich, X.Cui, E.Berk and **V.Muzykantov**. ICAM-1 targeted nanocarriers directed to endothelial cells. *Ibid*, page 94
 32. M.Christofidou-Solomidou, V.Shuvaev, S.Tliba, E.Arguiri, C.Solomides and **V.Muzykantov**. Targeting of catalase to endothelium reduces pulmonary thrombosis induced by acute oxidative stress in mice. *Abstr. ATS International Conference, May 20-25, 2005, San Diego, CA, Proc.Am.Thoracic Society, 2005, 2, p.A150*

D. 3. Unpublished presentations at scientific conferences and invited seminars.

Dr. V.Muzykantov presented the results of this project in the following invited lectures:

- 05/24/02 *Invited Speaker, ATS International Conference, Atlanta, GA:*
"Targeting antioxidant enzymes to the pulmonary vasculature"
- 07/10/02 *Invited Speaker, International Symposium "Reactive Oxygen Species",*
St.Petersburg, Russia,
"Intracellular delivery of catalase to endothelium"
- 09/31/02 *University of Cologne, Germany*
"Delivery of therapeutics to the pulmonary vasculature"
- 10/01/02 *University of Mainz, Germany*
"Targeting endothelial cell adhesion molecules"
- 10/03/02 *Urbino University, Italy*
"Novel strategies for vascular delivery of anti-thrombotic agents"
- 12/16/02 *Centocor, Radnor, PA*
"Perspectives for translation of the vascular immunotargeting into the clinical domain"
- 02/24/03 *Harvard University/MGH, Department of Radiology*
"Targeting of enzymes to surface adhesion molecules"

- 03/05/03 *Invited Speaker, International Symposium of Controlled Release and Advanced Drug Delivery Systems, Salt Lake City, Utah: "Vascular Immunotargeting of Antioxidant Enzymes to Endothelial Cells"*
- 04/04/03 *Department of Pharmacogenetics, Pittsburgh University, PA: "Delivery of antioxidants to pulmonary endothelium".*
- 06/24/03 *Department of Cardiology, Emory University School of Medicine, Atlanta, GA: "Targeting antioxidant enzymes via cell adhesion molecules"*
- 07/30/03 *Department of Pharmaceutical Sciences, University of Nebraska, Omaha: "Molecular design of drug delivery systems for targeting endothelium".*
- 08/10/03 *Invited Speaker, 6th World Congress on Inflammation (International Association Inflammation Societies Congress, IAIS), August 2-6th, Vancouver, Canada: "Targeting endothelial cell adhesion molecules"*
- 09/12/03 *Keynote Speaker, 4th Annual Colloquium "Cellular and Molecular Biomechanics", University of Virginia, Charlottesville, VA: "New horizons in targeting endothelial cell adhesion molecules"*
- 10/02/03 *Cardio-Pulmonary Research Institute, Winthrop University Hospital, SUNY at Stony Brook School of Medicine, Mineola, NY: "Novel strategies for protection against oxidant pulmonary stress".*
- 11/21/03 *Department Molecular Cardiology, Cleveland Clinic Foundation, Cleveland, OH: "Drug targeting to endothelial cells"*
- 01/21/04 *Invited Discussant, Transatlantic Airway Conference "Gene and Drug Therapies of Airway Diseases", Lucerne, Switzerland*
Drexel University, Department of Bioengineering, Philadelphia, PA: "Targeting endothelial cell adhesion molecules"
- 02/11/04 *Invited Speaker, **Gordon Research Conference** "Oxygen Radicals in Biology", Ventura, CA, February 8-13, 2004: "Targeting antioxidants to endothelium"*
- 04/10/04 *Drexel University, Department of Bioengineering, Philadelphia, PA: "Targeting therapeutics to ICAM-1"*
- 08/26/04 *Invited Speaker, **Gordon Research Conference** "Endothelial cell phenotypes in health and diseases", Andover, NH: "Targeted drug delivery to endothelium"*
- 10/06/04 *Department of Physiology, John Hopkins University, School of Health: "Delivery of antioxidant enzymes to pulmonary vasculature"*
- 11/03/04 *Division of Cardiovascular Medicine, University of Michigan, Ann Arbor, MI: "Endothelial cell adhesion molecules: therapeutic targets"*
- 11/16/04 *Department of Bioengineering, Ohio University, Athens, OH: "Delivery of anti-thrombotic agents to endothelial surface determinants"*
- 12/02/04 *Symposium of the University of Pennsylvania Institute for Medical Engineering: "Targeted drug delivery to defined cellular compartments in endothelial cells"*
- 01/28/05 *Department of Physiology, Case Western Reserve University: "Drug delivery to endothelial adhesion molecules"*
- 02/28/05 *Department of Medicine, Washington University Medical School, St.Louis, MO "Endothelial cell adhesion molecules: drug targets in the lungs"*
- 10/10/05 *Department of Organ Transplantation, University of Wisconsin, Madison, WI: "Targeting antioxidants to ICAM protects against pulmonary ischemia-reperfusion"*
- 11/30/05 *Department of Pharmaceutical Sciences, Groningen University, the*

Netherlands, "Drug targeting to cell adhesion molecules"

- 04/04/06 Annual FASEB Scientific Meeting (ASPET), San Francisco, CA: "Design of drug delivery to endothelial cells"
- 08/29/06 International Conference "Frontiers in Pharmacology", Chicago, IL: "Drug delivery to endothelium"
- 09/16/06 International PENN Workshop on Functional Lung Imaging, Philadelphia, PA: "Drug targeting to the pulmonary vasculature"
- 12/12/06 International Liposome Society Meeting, London, UK: "Nanocarriers for drug delivery into endothelial cells"

D.4. Graduate Students Training

Mr. Rudy Fuentes, a Graduate Student of the University of Pennsylvania Pharmacology Graduate Group, completed 2003 fall semester rotation training in the Muzykantov's lab. Mr. Fuentes studied effects of biotinylation and conjugation with anti-CAM on biochemical properties of SOD.

Ms. Anu Thomas, a Graduate Student of the Philadelphia University of Science, enlisted for her Ph.D. Thesis experimental studies in Muzykantov's lab since fall semester of 2004. Ms. Thomas studies mechanisms of internalization of anti-CAM conjugates in endothelial cells.

Mr. Armen Karamajan, a Graduate Student of the University of Pennsylvania Pharmacology Graduate Group, completed 2004 fall semester rotation training in the Muzykantov's lab. His rotation project involved experiments with flow-adapted endothelial cells.

Ms. Weining Qui, a Graduate Student of the University of Pennsylvania Bioengineering Graduate Group, completed 2004 summer-fall semesters rotation training in Muzykantov's lab. Her rotation projects involved quantitative analysis of binding anti-ICAM conjugates to endothelial cells. She passed the first qualifying exam and currently works on Ph.D. thesis proposal on analysis of anti-ICAM conjugates binding to endothelium in Muzykantov's lab.

Mr. Bisen Ding, a Graduate Student of the of the University of Pennsylvania Pharmacology Graduate Group, completed 2004 fall semester rotation training in the Muzykantov's lab. Mr Ding is a key researcher involved in design and testing of recombinant fusion anti-CAM scFv-scuPA constructs and pursues his Ph.D. project on targeted delivery of anti-thrombotic agents in Muzykantov's lab.

Ms. Qui and Mr. Ding completed their rotations and course work in Graduate Groups of Bioengineering and Pharmacology, respectively, and embarked on their Ph.D. Thesis projects in Muzykantov's lab, followed their directions of research outlined above.

Ms. Julia Tsurpina, a graduate student in Bioengineering Department of University of Pennsylvania School of Engineering and Applied Science, completed her rotation in Muzykantov's lab. Her research project was focused on characterization of anti-CAM/SOD conjugates in cell cultures.

Ms. Allison Leshner, a graduate student in Pharmacology, completed her fall semester rotation in Muzykantov's lab. Her project was focused on detection of intracellular ROS in EC and their decomposition by anti-CAM/AOE.

Ms. Lindsay McKenna, a graduate student in Pharmacology, completed her fall semester rotation in Muzykantov's lab. Her project was focused on the molecular signaling mechanisms involved in CAM-mediated endocytosis.

Mr. Bi-Sen Ding, graduate student in Pharmacology, had his Ph.D. Thesis Prelim Exam approved, works on his Thesis research project in Muzykantov's lab and expected to prepare dissertation for the defense in 2008. His project is focused on design and testing of scFv/PA fusion proteins.

D.5. Special Honors and Recognition by the Scientific Community.

- 1. Dr. Muzykantov has been invited to the **Gordon Research Conference** "Oxygen Radicals in Biology" (Ventura, CA, February 8-13, 2004) to give a talk: "Targeting antioxidant enzymes to vascular endothelium". Traditionally, talks at GRC (that is regarded as the highest-level scientific forum) are not published in order to permit unlimited sharing of fresh research data with peers.*
- 2. Dr. Muzykantov organized and chaired a Symposium on Targeted Drug Delivery at 6th World Congress on Inflammation (International Association Inflammation Societies Congress, IAIS), August 2-6th, Vancouver, Canada*
- 3. Dr. Muzykantov has been invited as a Keynote Speaker to 4th Annual Colloquium "Cellular and Molecular Biomechanics", University of Virginia, Charlottesville, VA (September 12th, 2003).*
- 4. Dr. Muzykantov has been invited to give a talk "Targeted drug delivery to endothelium" at the **Gordon Research Conference** "Endothelial cell phenotypes in health and diseases", Andover, NH, August 22-27, 2004.*
- 5. Dr. Muzykantov served as a chair of session on targeted drug delivery at the Gordon Research Conference on Drug Delivery, Big Sky, MT, 09/08/04*
- 6. Dr. Muzykantov has been invited as a featured speaker and the master class moderator to a Graduate Course in Drug Delivery in University of Groningen, The Netherlands, November 30th -December 4th, 2005*
- 7. Dr. Muzykantov served as a member of the Organizing Committee and session Chair for the International PENN Workshop on Functional Lung Imaging, 09/14-16/06, Philadelphia, PA*
- 8. Dr. Muzykantov served as Chair of Organizing Committee, University of Pennsylvania Targeted Therapeutics Retreat, 11/16/06, Philadelphia, PA*

E. Conclusions.

Systematic characterization and optimization of design of a series of CAM-targeted AOE conjugates and PA fusion proteins is successfully completed and showed high potential for industrial development, translation and clinical utility of these novel agents for targeting AOE and PA to endothelial cells.

We have discovered size-controlled CAM-mediated endocytosis of anti-CAM conjugates and characterized molecular and cellular mechanisms that control this process (NHE-1, actin-mediated and dynamin controlled, PKC- and calcium coupled activation of plasma membrane vacuolization).

We have characterized intracellular traffic, including NHE-1/NHE-6 endosome/lysosome switch, CAM dissociation and recycling to the EC surface, final lysosomal destination, metabolism and duration of anti-CAM conjugates internalized by EC and defined auxiliary drugs, which in a controlled fashion alter intracellular traffic or lysosomal degradation, markedly prolongs duration and activity of internalized anti-CAM conjugates.

We quantitatively characterized targeting of anti-CAM and anti-CAM conjugates of diverse size and shape in intact animals and in animals with models of acute lung injury, defined optimal size (~300 nm diameter) providing the best specificity characteristics of the targeting and found that vascular pathology augments CAM-directed pulmonary targeting.

We have quantitatively characterized proposed animal models of ALI (anti-TM/GOX or/and hyperoxia) using specific parameters of lung injury and CAM expression on EC. We studied role of WBC in these models of ALI by blocking interactions of WBC with CAM and WBC elimination and found that WBC efflux into the lung provides only little, if any, augmentation of the injury.

We designed and produced anti-CAM/AOE conjugates of optimal size, enzymatic activity and affinity to EC, which accumulate in the pulmonary vasculature after intravenous injection in mice.

We have established a series of cellular model of endothelial injury caused by extracellularly and intracellularly produced ROS and found that anti-CAM/SOD provides rather partial protection in all models, while anti-CAM/SOD-catalase tandem conjugate provides complete protection.

In animal models of vascular oxidative stress, anti-CAM/catalase, but not anti-CAM/SOD was protective in anti-TM/GOX model (implicating the primary role of H₂O₂), whereas anti-CAM/SOD, but not anti-CAM/catalase, was protective in Ang-II vasoconstriction model (implicating the primary role of superoxide).

We found that CAM-targeted plasminogen activator resides in the luminal surface and facilitates fibrinolysis of emboli lodged in the pulmonary vessels. Using in vitro and in vivo studies of fibrinolysis of plasma clots, we have selected a candidate constitutive fibrinolytic agent (Tenektase and urokinase, respectively)

and using a recombinant gene engineering techniques we designed a series of new fusion protein constructs, plasmin activated scFv-uPA and thrombin activated scFv-scFvPA-T. Both constructs bind to EC, accumulate in the lungs and stimulate subsequent fibrinolysis of thrombi in lungs, yet scFv-uPA-T provides more durable and safe effect. Anti-CAM scFv-scFvPA-T is a candidate drug has extremely good chances for swift translation into clinical domain, in particular, for alleviation of thrombotic pathological component of ALI.

Pilot studies in the mouse in situ lung ischemia-reperfusion model established in the lab showed that both anti-CAM/AOE and scFv/uPA-T protect the lung from oxidative and thrombotic injury and augment blood oxygenation.

The general conclusion is that the achieved research progress: i) corresponds to the SOW and research plan projected for the grant; ii) indicates that all Specific Aims (Tasks) are currently completed; and; iii) provides a solid basis for translation of a new strategy, namely, targeting of antioxidant and anti-thrombotic enzymes to endothelial cell adhesion molecules, into pre-clinical and ultimately clinical domain, for improved management of ALI/ARDS and, likely other vascular and pulmonary syndromes coupled with oxidative stress, thrombosis, inflammation and ischemia.

G. References. In this section we provide exclusively references to the published studies from our group. All references to other publications pertinent to this report can be found in the attached appendix items.

1. V.Shuvaev, T.Dziubla, R.Wiewrodt, and **V.Muzykantov*** (2004) Streptavidin-biotin cross-linking of therapeutic enzymes with carrier antibodies: nanoconjugates for protection against endothelial oxidative stress. "Methods in Molecular Biology. Bioconjugation Protocols: Strategies and Methods", C.M.Niemeyer, Ed., Humana Press, Totowa, NJ, Chapter 1, pp 3-18
2. R.Wiewrodt, A.Thomas, L.Cipelletti, M.Christofidou-Solomidou, D.Weitz, S.I.Feinstein, D.Schaffer, S.M.Albelda, M.Koval and **V.Muzykantov*** (2002) Size-dependent immunotargeting of cargo materials into endothelial cells. *Blood*, 99: 912-922
3. S.Muro, R.Wiewrodt, A.Thomas, L.Koniaris, S.Albelda, **V.Muzykantov*** and M.Koval (2003) A novel endocytic pathway induced by clustering endothelial ICAM-1 or PECAM-1. *J Cell Sci.* 2003 Apr 15;116(Pt 8):1599-1609
4. S.Muro, X.Cui, C.Gajewski, **V.Muzykantov*** and M.Koval (2003) Slow intracellular trafficking of catalase nanoparticles targeted to ICAM-1 protects endothelial cells from oxidative stress. *Am.J.Physiol., Cell Physiol.*, 285(5):C1339-47
5. S.Muro, M.Matescu, C.Gayewsky, **V.Muzykantov*** and M.Koval (2006) Control of intracellular trafficking of ICAM-1 targeted nanocarriers by endothelial Na/N exchanger (NHE) proteins. *Am.J.Physiol. Lung Cell Mol.Physiol.*, 290:L809-817
6. S.Muro, C.Gayewsky, M.Koval and **V.Muzykantov** (2005) ICAM-1 recycling in endothelial cells: a novel pathway for sustained intracellular delivery and prolonged effects of drugs. *Blood*, 105:650-658
7. S.Muro, **V.Muzykantov*** and J.Murciano (2004) Characterization of endothelial internalization and targeting of antibody-enzyme conjugates in cell cultures and in laboratory animals "Methods in Molecular Biology. Bioconjugation Protocols: Strategies and Methods", C.M.Niemeyer, Ed., Humana Press, Totowa, NJ, Chapter 2, pp 21-36
8. S.Muro, M.Koval and **V.Muzykantov*** (2004) Endothelial endocytic pathways: gates for vascular drug delivery. *Current Vascular Pharmacology*, 2:281-299
9. S.Muro and **V.Muzykantov*** (2005) Targeting of antioxidant and anti-thrombotic drugs to endothelial cell adhesion molecules. *Current Pharmacological Design*, 11, 2383-2401
10. J.C.Murciano, S.Muro, L.Koniaris, M.Christofidou-Solomidou, D.Harshaw, S.Albelda, D.Granger, D.Cines and **V.R.Muzykantov*** (2003) ICAM-directed vascular immunotargeting of anti-thrombotic agents to the endothelial luminal surface. *Blood*, 101:3977-1984
11. S.Perkowski, A.Scherpereel, J-C.Murciano, E.Arguiri, C.Solomides, S.Albelda, **V.R.Muzykantov** and M.Christofidou-Solomidou (2006) Dissociation between alveolar transmigration of neutrophils and lung injury in hyperoxia. *Am.J.Physiol. Lung Cell Mol.Physiol.*, 291(5):L1050-8

12. V.Shuvaev, M.Christofidou-Solomidou, A.Scherpereel, E. Simone, E.Arguiri, S.Tliba, J.Pick, S.Kennel, S.Albelda and **V.R.Muzykantov*** (2007) Factors modulating the delivery and effect of enzymatic cargo conjugated with antibodies targeted to the pulmonary endothelium *J.Controlled Release*, 118:235-244
13. Bi-Sen Ding, C.Gottstein, A.Grunow, A.Kuo, K.Ganguly, S.Albelda, D.Cines and **V.R.Muzykantov*** (2005) Endothelial targeting of a recombinant construct fusing a PECAM-1 single-chain variable antibody fragment (scFv) with pro-urokinase facilitates prophylactic thrombolysis in the pulmonary vasculature. *Blood*, 106:4191-4198

Appendices

Attached are key publications from our lab used as the source of references to specific results listed in the report:

1. V.Shuvaev, T.Dziubla, R.Wiewrodt, and **V.Muzykantov*** (2004) Streptavidin-biotin cross-linking of therapeutic enzymes with carrier antibodies: nanoconjugates for protection against endothelial oxidative stress. "Methods in Molecular Biology. Bioconjugation Protocols: Strategies and Methods", C.M.Niemeyer, Ed., Humana Press, Totowa, NJ, Chapter 1, pp 3-18
2. S.Muro, R.Wiewrodt, A.Thomas, L.Koniaris, S.Albelda, **V.Muzykantov*** and M.Koval (2003) A novel endocytic pathway induced by clustering endothelial ICAM-1 or PECAM-1. *J Cell Sci.* 2003 Apr 15;116(Pt 8):1599-1609
3. S.Muro, X.Cui, C.Gajewski, **V.Muzykantov*** and M.Koval (2003) Slow intracellular trafficking of catalase nanoparticles targeted to ICAM-1 protects endothelial cells from oxidative stress. *Am.J.Physiol., Cell Physiol.*, 285(5):C1339-47
4. S.Muro, M.Matescu, C.Gayewsky, **V.Muzykantov*** and M.Koval (2006) Control of intracellular trafficking of ICAM-1 targeted nanocarriers by endothelial Na/N exchanger (NHE) proteins. *Am.J.Physiol. Lung Cell Mol.Physiol.*, 290:L809-817
5. S.Muro, C.Gayewsky, M.Koval and **V.Muzykantov** (2005) ICAM-1 recycling in endothelial cells: a novel pathway for sustained intracellular delivery and prolonged effects of drugs. *Blood*, 105:650-658
6. J.C.Murciano, S.Muro, L.Koniaris, M.Christofidou-Solomidou, D.Harshaw, S.Albelda, D.Granger, D.Cines and **V.R.Muzykantov*** (2003) ICAM-directed vascular immunotargeting of anti-thrombotic agents to the endothelial luminal surface. *Blood*, 101:3977-1984S.
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8. V.Shuvaev, M.Christofidou-Solomidou, A.Scherpereel, E. Simone, E.Arguiri, S.Tliba, J.Pick, S.Kennel, S.Albelda and **V.R.Muzykantov*** (2007) Factors modulating the delivery and effect of enzymatic cargo conjugated with antibodies targeted to the pulmonary endothelium *J.Controlled Release*, 118:235-244
9. S.Muro, T.Dziubla, W.Qui, J.Lefterovich, X.Cui, E.Berk and **V.R.Muzykantov*** (2006) Endothelial targeting of high-affinity multivalent polymer nanocarriers directed to ICAM-1. *J.Pharm.Exp.Therap.*, 317:1161-1169S.
10. Perkowski, A.Scherpereel, J-C.Murciano, E.Arguiri, C.Solomides, S.Albelda, **V.R.Muzykantov** and M.Christofidou-Solomidou (2006) Dissociation between alveolar transmigration of neutrophils and lung injury in hyperoxia. *Am.J.Physiol. Lung Cell Mol.Physiol.*, 291(5):L1050-8

11. V.Shuvaev, S.Tliba, M.Nakada, S.Albelda and **V.R.Muzykantov*** (2007) PECAM-directed endothelial targeting of SOD alleviates oxidative stress caused by either extracellular or intracellular superoxide. *J.Pharm.Exp.Therap.*, 323, in press
12. Bi-Sen Ding, C.Gottstein, A.Grunow, A.Kuo, K.Ganguly, S.Albelda, D.Cines and **V.R.Muzykantov*** (2005) Endothelial targeting of a recombinant construct fusing a PECAM-1 single-chain variable antibody fragment (scFv) with pro-urokinase facilitates prophylactic thrombolysis in the pulmonary vasculature. *Blood*, 106:4191-4198